The application of ultrasound in the bioconversion of brewer's and distiller's spent grains to bioethanol

Jason Bennett

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I certify that this is the true and accurate version of the thesis approved by the examiners

Signed..... **Date.....**

Director of Studies

Abstract

In recent years bioethanol (fuel ethanol derived through fermentation) has become the leading alternative to currently utilised liquid transportation fuels, possessing the benefits of being both sustainable and carbon neutral. If this position is to be maintained, it is clear that the feedstocks and processes used during its production have to be a major focus for the future direction of research, not only within the context of bioethanol but all biofuels in general. In terms of future sustainability, it is important that biofuel production should be derived from waste materials which exhibit limited potential for use in other applications. In this manner, food-to-fuel debates can be circumvented.

Lignocellulose represents a readily available biowaste material for biofuel generation. The brewing and distilling industries in particular are uniquely placed to exploit the conversion of lignocellulose to bioethanol, through the utilisation of the millions of tons of spent grains produced annually by distilleries across the globe. Conversion of spent grains (SG) to bioethanol represents one of the most attractive and indeed challenging opportunities for meeting demand for sustainable bioethanol production. However it presents considerable challenges in terms of costs of production and associated energy balances. Enzyme costs are a particularly challenging issue and any technological developments which have potential to increase cellulolysis and decrease cellulase enzyme dosage are of distinct importance. Additionally, current pre-treatment approaches rely heavily of the use of acids during the pre-treatment stage. This generates a variety of compounds which are inhibitory to fermentation (e.g. acetic acid, furfural) and lead to low ethanol yields. Research which can reduce enzyme loading or improve low temperature pre-treatment are of significant importance.

The research described here sought to evaluate the use of ultrasound in the pre-treatment and enzymolysis of spent grains. The use of acid in the pre-treatment and enzymolysis of SG was optimised to provide a bench-mark for novel hydrolysis techniques. Ultrasound (20 kHz) was assessed as a pre-treatment technique in its own right as well as in combination with various oxidising chemicals such as ozone and hydrogen peroxide. Additionally, high frequency ultrasound (>500 kHz) was investigated in terms of its effect upon the activity of the enzymes involved in SG hydrolysis. A number of yeast species (*S.cerevisiae, P.stipitis, K.marxianus, P. tannophilus* and *C. shehatae*) were then evaluated for their ability to ferment the mix of five and six carbon sugars liberated during enzymatic hydrolysis of SG.

Ultrasound was found to be effective in enhancing combined ozone and hydrogen peroxide pre-treatment of SG, although not as effective as the use of acid. Additionally, ultrasound was shown to significantly enhance the activity of cellulose, xylanase and β- glucosidase, with the magnitude of the increase highly dependent on frequency and output power. The research presented here has expanded knowledge in terms of the effect of ultrasound upon lignocellulose as well as the enzymes involved in its digestion.

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Introduction

1. Introduction

1.1 Peak oil, the energy crisis and the need for sustainable energy

Global dependence upon fossil fuel reserves is well documented, with much of our modern way of life dependent on oil, coal or gas in one way or another. Activities that rely on fossil fuels range from transportation and agriculture through to electricity generation and modern medicine. Due to this we are faced with an unprecedented problem that will manifest itself more and more in the coming decades.

The finite nature of the energy supplies upon which humanity relies is a well-established phenomenon, with various studies concluding that global oil production will peak somewhere between 1996 and 2035 (Demirbas, 2008). The notion of peak oil or Hubbert peak theory was initially proposed in the 1950's by Marion Hubbert, who at the time was a geoscientist working for the large multinational oil corporation Royal Dutch Shell. It is defined as the point in time at which global oil extraction hits its peak and eventually enters terminal decline (Almeida and Silva, 2009).

More recent studies have determined that whilst data for oil reserve growth is encouraging, it should be viewed within a context that it is likely two thirds of the oil fields upon which current production relies may have been depleted by 2030 (Sorrell *et al.,* 2011.) Whatever the eventual time-frame during which global oil production grinds to a halt, it is clear that future energy policy should seek to replace fossil fuel derived sources of energy, with those of a more sustainable and environmentally friendly nature.

Sustainability has become something of a “buzz word” within both a scientific and industrial circles. Its exact definition is highly subjective (Holling, 2000) and varies depending upon the context in which it is applied. However, it appears to be case that sustainability with regards to human development refers to a way of working which satisfies environmental concerns as well those of a more traditional nature (e.g. social and economic). Sustainability refers to the need for human development to proceed without having a detrimental effect upon planet earth. In terms of energy generation, sustainability refers to methods of energy production that are renewable and do not deplete fossil fuel resources.

Renewable sources of energy have come under increasing focus during the last decade. These sources come in various guises including; wind, solar, tidal, wave, biofuels and biomass. In terms of replacement of fossil fuel derived sources of transportation fuel, biofuels are touted as one avenue which can be exploited.

1.2 Biofuel characteristics

A biofuel is defined as any source of energy (either liquid, gas or solid) which is derived through the conversion or combustion of a renewable biological feedstock. In contrast with other types of renewable (e.g. wind, tidal and solar energy) biofuels can be used as direct replacements for conventional liquid transportation fuels currently used in combustion engines. Numerous biofuel types exist. However, the most heavily developed are biomethane, biobutanol, biohydrogen, biodiesel and bioethanol. Of the five main classes of biofuel, biomethane, biodiesel and bioethanol are most developed. Large

scale industrial production of both 1st generation bioethanol and biodiesel are a reality and as such they are arguably of greater current importance than either biobutanol or biohydrogen.

Biofuels are deemed to have many advantages over traditional fossil fuels, in that they are sustainable, may help to reduce green house gases and, if produced domestically, lead to increase in the security of energy supply (Reijnders, 2006). However, since their widespread implementation, biofuels have received significant negative press within the mainstream media. Many of these concerns focus on the so called "food to fuel" debate (Zhang, *et al.*, 2010; Valentine, *et al.*, 2012; Anderson, 2012) which questions the moral and ethical issues surrounding devoting thousands of acres of agricultural land are devoted to producing energy crops rather than food.

1.2.1 Biomethane

Biomethane is usually produced through the bioconversion of organic wastes via a four stage anaerobic process: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Korres *et al.*, 2010) with each of the stages being carried out by different microbial populations within the bioreactor. The initial stage of anaerobic digestion involves the hydrolysis of the large organic polymers within the biomass. The breakdown is carried out by endogenous microbial enzymes which act upon biomass carbohydrates, fats and proteins yielding a variety of sugars, fatty acids and amino acids, respectively (Asam *et al.*, 2011). During the second stage, acidogenic bacteria convert the products of hydrolysis into a variety of compounds including volatile fatty

acids, alcohols, hydrogen and carbon dioxide. The principal products formed during this stage are propionic, butyric, acetic, formic and lactic acids alongside ethanol, methanol, hydrogen and carbon dioxide. Acetogenesis then converts the propionic and butyric acids, produced during acidogenesis, along with the alcohols into hydrogen, carbon dioxide and acetic acid. The final stage in anaerobic digestion, known as methanogenesis, involves the conversion of the hydrogen and acetic acid formed during stages two and three, into methane and carbon dioxide (Korres *et al*., 2010). The resultant biogas is then captured and usually combusted at the site of production as the feed to a combined heat and power plant (CHP) or exported directly to the grid.

1.2.2 Biohydrogen

Conventional methods for the production of industrial hydrogen usually involve either the electrolysis of water or steam reformation of methane (Levin *et al.,* 2004). Biohydrogen differs to traditionally produced hydrogen in that it is synthesized during the metabolic processes of a variety of microorganisms. The production of biohydrogen usually harnesses one of three biological processes, these being bio photolysis, photo fermentation, or dark fermentation.

1.2.3 Biodiesel

Biodiesel is characterised as being monoalkyl esters derived from plant oils or animal fats (Demirbas, 2007). During biodiesel production, the viscous nature of the feedstock, requires the transesterification of the oil or fat, which brings the viscosity in line with that of petroleum based diesel fuel. The

process involves the treatment of the fat or oil with a monohydric alcohol (usually methanol, ethanol or butanol), whilst in the presence of a catalyst (usually sodium hydroxide, potassium hydroxide or sulphuric acid). The conversion results in triglycerides reacting with the monohydric alcohol to form glycerin and monoalkyl esters (biodiesel). Feedstocks that can be utilised in the production of biodiesel are varied and include: palm oil, peanut oil, sunflower oil, rape-seed oil, soya bean oil, animal tallow and waste cooking oils. Biodiesel can be used as a direct replacement for biodiesel without existing engine modifications.

1.2.4 Biobutanol

Butanol or butyl alcohol is a four carbon alcohol with the formula C_4H_9OH. As is the case with ethanol it is a volatile flammable liquid that can be used as an alternative to conventional fossil fuel derived liquid transportation fuels. Traditionally butanol has been derived commercially from fossil fuels through hydroformylation of propene to butyraldehyde which is subsequently reduced with hydrogen to butanol (Green, 2011). Biobutanol differs from conventional butanol in that it is derived through the bioconversion of biomass and as such is deemed to be renewable.

Typically biobutanol is produced through the conversion of biomass sugars to butanol utilising bacterial acetone-butanol-ethanol (ABE) fermentation, with feed-stocks usually being either starch (Al-shorgani, Kalil and Yusoff, 2012) or cellulose based (Ranjan, Khanna and Moholkar, 2013). The process usually involves *Clostridium spp*. bacteria (e.g *Clostridium acetobutylicum*) which ferment biomass sugars in an anaerobic fermentation

similar to yeast fermentation, with products produced at a ratio of ~ 3:6:1 (acetone: butanol: ethanol) (Garcia *et al.*, 2011).

It is anticipated that biobutanol may eventually become a more attractive replacement for liquid transportation fuels than bioethanol. Biobutanol displays a number of advantages over ethanol including having a higher energy content and lower water absorption (Durre, 2007). However, biobutanol is seriously disadvantaged by a number of factors, specifically extremely low yields, which in turn increases feedstock costs and leads to energy intensive distillation (Green, 2011). As such it is likely that bioethanol will remain the biofuel of choice in terms of replacement of conventional unleaded gasoline for the foreseeable future.

1.2.5 Bioethanol

Ethanol or ethyl–alcohol is a two carbon alcohol with the formula C_2H_5OH (Fig. 1.1). It is a volatile flammable liquid that is increasingly being used as an alternative to fossil fuel derived transportation fuels. Ethanol was used as a liquid transportation fuel as far back as 1908 when Henry Ford designed his model T to run on alcohol. Ethanol can be used as the sole fuel in a combustion engine or blended with traditional petroleum based fuels (Gray, Zhao and Emptage, 2006). Ethanol, when used as a transportation fuel, has a number of advantages when compared to conventional petroleum based fuels (Table 1.1), in that it has a higher octane rating, lower auto ignition temperature and reduced lower heating value than conventional gasoline (Walker, 2010). This leads to increased engine power output and reduced heat and wear.

Table 1.1 Comparative characteristics of alcohol fuels, isooctane and conventional unleaded gasoline (data from Walker, 2010)

Fuel property	Isooctane	Methanol	Ethanol	Unleaded gasoline
Octane number	100	112	107	97
Auto-ignition temperature (K)	530	737	606	643
Latent heat of vaporization (MJ/Kg)	0.26	1.18	0.91	0.35
Lower heating value (MJ/Kg)	44.4	19.9	26.7	43.1

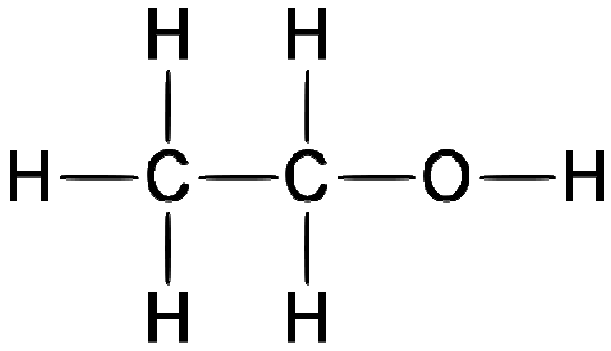


Fig 1.1 – Molecular structure of ethanol

Bioethanol represents one of the most sustainable alternatives to existing fuels, exhibiting the added benefits of being both renewable (Balat *et al*., 2008.) and environmentally friendly (Baras *et al*., 2002). It is regarded as renewable due to being derived from plant based feedstocks which can be replenished by modern agriculture (McMillan, 1997). This in turn leads to bioethanol being considered as environmentally friendly as it does not deplete non-renewable fossil fuel resources. Additionally, bioethanol is regarded as being carbon neutral (Kumar *et* al., 2009) as the CO_2 emitted during combustion is offset by the CO_2 consumed during photosynthetic growth of agricultural biofuel feedstocks. There is however a current debate within the scientific community as to the relative CO_2 savings observed with 1st generation bioethanol compared to fossil fuel derived transportation fuels (discussed in detail later in this chapter).

Bioethanol is fuel ethanol derived through fermentation, with the sugars consumed during fermentation coming from a biomass based feedstock. Sources of biomass that can be used in the production of bioethanol come in a variety of forms including starch, fructose, sucrose and cellulose based biomass. This contrasts with traditional industrial ethanol, which is synthesised during crude oil fractionation.

The process stages in the production of bioethanol are closely aligned to those implemented by the brewing and distilling industries during the production of potable alcoholic beverages. The first stage in the process involves extraction of sugars from the designated biomass feedstock (e.g. maize or sugar cane). Regardless of the biomass to be used this is usually done enzymatically, although certain types of biomass require a prior pre-

treatment stage. Following the extraction of carbohydrate, the sugars produced are fermented, usually by yeast, to produce ethanol. The ethanol generated during fermentation is distilled to produce bioethanol. The processes involved in the production of bioethanol will be examined in detail later in this chapter.

1.3 Feedstocks and processes for bioethanol production

Feedstocks that can be utilised in the production of bioethanol are diverse and varied, being classified as either 1st, 2nd or 3rd generation and usually comprise four types – starch, sugar, lignocellulose or algae based (Balat *et al*., 2008).

1.3.1 First generation feedstocks

1st generation feedstocks are defined as plant biomass that has been grown specifically for the production of bioethanol. Such biomass can also be used for other purposes, particularly as a human or animal food sources. They usually fall into two broad categories, being either starch or sugar based.

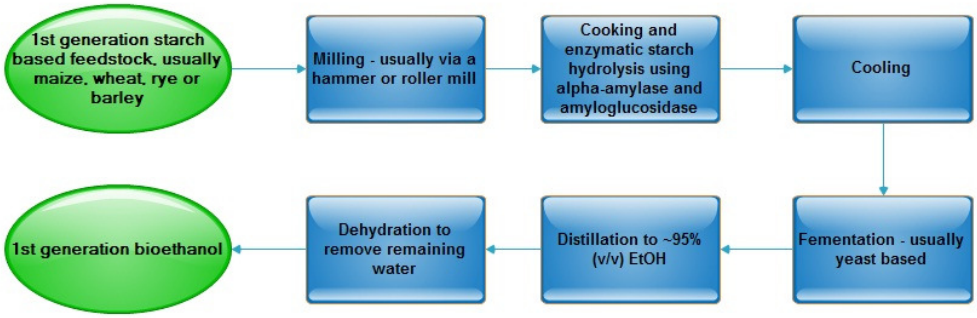
1.3.1.1 Starch based

Starch based feedstocks usually come in the form of either the cereal grains - maize (Kim and Dale, 2002), wheat (Dong *et al*., 2008), barley (Gibreel *et al*., 2009), rye (Wang *et al*., 1998), triticale (Kucerova, 2007) or the root crops potato (Tasic *et al*., 2009) and cassava (Ziska *et al*., 2009). The generic process stages in the production of bioethanol from starch based feedstocks are summarised in Fig. 1.2a. A typical dry grind process used for maize in the United States consists of five stages: milling, cooking and enzymatic starch hydrolysis, distillation to produce hydrous ethanol at ~ 95% (v/v) and dehydration to produce anhydrous bioethanol (Kwiatkowski *et al*., 2006).

1.3.1.2 Sugar based

Sugar based feedstocks differ from starch based feedstocks in that the sugars utilised during bioethanol production come in an already accessible form and do not require the hydrolysis of carbohydrate to monomeric fermentable sugars. Sucrose-based sugar cane (Dias *et al.*, 2011) and sugar beet (Mantovani, 2006) are the principal feedstocks of this type. The process stages in the production of bioethanol from sugar based feedstocks are summarised in Fig. 1.2b. In Brazil, processing differs dependent on whether or not the sucrose contained within sugar cane is to be used to produce sugar or bioethanol. If bioethanol is the solely desired product, the process consists of five stages: pressing, water addition, fermentation, distillation to produce hydrous ethanol at ~ 95 % (v/v) and dehydration to produce anhydrous bioethanol. If the sucrose contained within sugar cane juice is to be diverted to sugar production the process differs in that the juice is evaporated to produce sucrose crystals and molasses. The molasses, containing glucose, fructose and varying levels of sucrose (dependent on the amount crystalised during evaporation) provide the medium for bioethanol fermentation.

(a)



(b)

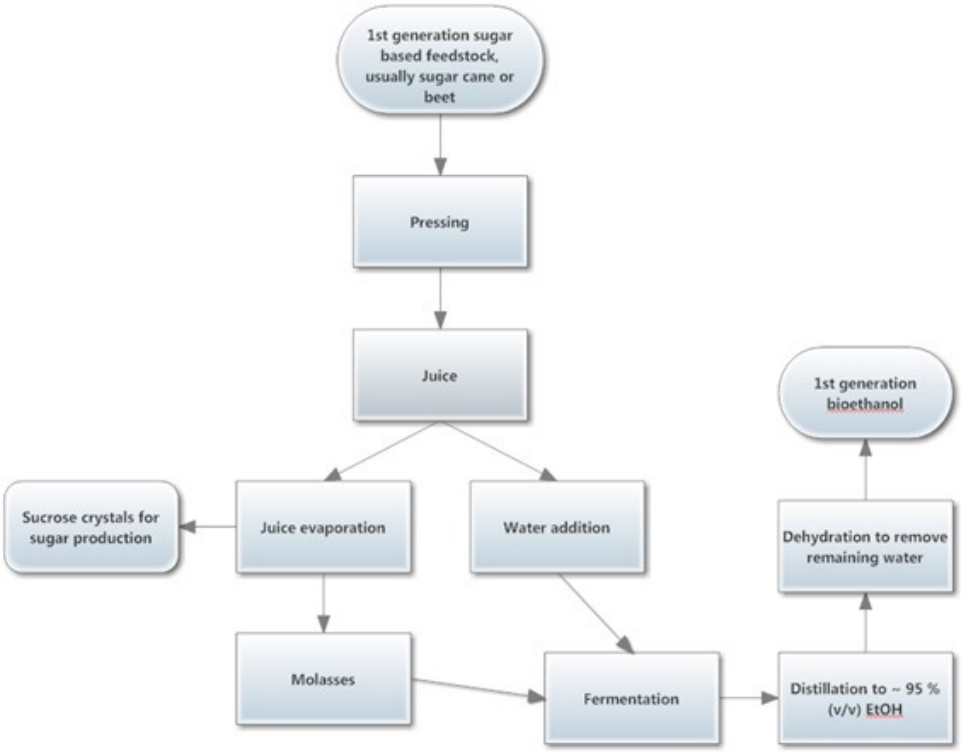


Fig 1.2 Process stages in the production of bioethanol from 1st generation feedstocks: (a) starch based (b) sugar based

1.3.2 Second generation feedstocks

Bioethanol feedstocks that are non-food based are termed 2nd generation, with the main source of biomass of this type being lignocellulose, which represents one of the most abundant sources of carbon on earth. Primary sources of lignocellulose classed as being energy crops (e.g. Miscanthus grass) or waste materials. Potential sources of waste lignocellulose are vast and include; straw, woody wastes from forestry, waste paper and cardboard, agricultural residues, corn residues and waste from the brewing and distilling industries (e.g. spent grains). The processes used in the production of bioethanol from lignocellulose are significantly more complex than those used in the conversion of 1st generation feedstocks. These processes are discussed in detail later in this chapter.

1.3.3 Third generation feedstocks

Third generation bioethanol is not well defined, in that the substrates and processes used in its production are not universally accepted. Third generation bioethanol usually refers to fuel ethanol derived from microalgae (Harun *et al*., 2011). The production processes are similar in that the biomass must be hydrolysed and the extracted sugar fermented to produce bioethanol. However it is becoming apparent that the use of microalgae as a feedstock for biofuel production is more suited to the production of biodiesel due to its high lipid content (Scott *et al*., 2010).

1.4 Drivers in the development of bioethanol for transportation

Sustainable alternatives to transportation fuels are highly dependent on new and existing biofuel technologies. In recent years factors driving the

development and commercial implementation of sustainable and economically competitive alternatives to liquid transportation fuels have increased considerably. More specifically, legislative CO_2 reduction targets designed to mitigate the effect of anthropogenic climate change, in conjunction with diminishing fossil fuel resources, have concentrated efforts within the research community to develop sustainable, technically feasible and carbon neutral biofuel replacements to traditional oil based transport fuels. It is becoming increasingly clear that within decades, alternatives to dwindling liquid transportation fuels are going to need to become main-stream.

In light of this governments around the world have sought to drive bioethanol production (alongside other biofuels), using a variety of international treaties alongside domestic legislation. The Kyoto agreement of 1997 (UN Doc FCCC/CP/1997/7/Add.1) obligates signatories to the agreement, to cut their CO_2 emissions by an average of 4.8 %, during 2008-2012, using 1990 emissions levels as a baseline. Emissions reductions targets vary by country, with the UK agreeing to a decrease of 12.5 %, compared to an EU average of 8 %. It is of note that, at the time of writing, the world's largest producer of CO_2 emissions, the United States, is not a signatory to the agreement (UN Doc FCCC/CP/1997/7/Add.1).

However, a number of legislative drivers exist within the United States to drive bioethanol production. The American Energy Policy Act of 2005 (U.S. Government Printing Office, 2005) initiated the Renewable Fuel Standard (RFS), which requires gasoline producers to use an increasing blend of biofuels, such as bioethanol and biodiesel. Coupled with this the act

created a number of incentives for the production of bioethanol from both sugar and cellulose based feedstocks. There are a wide variety of cellulose based feedstocks that are suitable for conversion to bioethanol. These mainly come in the form of lignocellulosic plant residues such as corn stover (Agbogbo and Wenger, 2007), wheat straw (Saha *et al*., 2005) and spent grains (White *et al*., 2008). Additional sources of lignocellulose for bioethanol production come in the form of purpose grown energy crops such as Miscanthus grass (Heaton, Dohleman and Long, 2008).

The Energy Policy act 2005 was further developed by the Energy Independence and Security Act of 2007 (U.S. Government Printing Office, 2007), which set targets for year on year increases in the volumes of renewable fuels being utilised. These targets required the use of 40.91 billion litres in 2008, rising to 72.74 billion litres in 2012 and 138.2 billion litres by 2022. The act went further, in that it set individual maxima for the production of fuels from corn, sugar cane and cellulosic feedstocks.

In terms of the EU, a number of treaties relate to renewable energy in general and various legislative directives incentivise biofuel production. The directive on the promotion of the use of biofuels and other renewable fuels for transport (Council Directive 2009/28/EC), stipulates that by 2020, member states must be sourcing 20% of their general energy requirements from renewable resources, with an additional proviso that 10% of transportation energy must also be renewable. Against a backdrop of grain mountains and common agricultural policy (CAP) payments to farmers for set aside land, the Biofuels Directive 2003 (Council Directive 2003/30/EC), set minimum levels for biofuel penetration of 2 % by the end of 2005 and 5.75 %

by the end of 2010. However, this act was subsequently repealed and replaced by Council Directive 2009/28/EC. The new act, whilst still placing an important emphasis on the need for increased adoption of biofuel for transportation, called for "an assessment of the feasibility of reaching the target whilst ensuring the sustainability of biofuels production in the Community and in third countries, and considering economic, environmental and social impacts, including indirect effects and impacts on biodiversity, as well as the commercial availability of second-generation biofuels" (Council Directive 2003/30/EC). It further clarified the stance of the EU in terms of 2nd generation biofuels by asking for further assessment into "the availability of biofuels made from waste, residues, non-food cellulosic material and lignocellulosic material" (Council Directive 2003/30/EC).

UK biofuel implementation is governed by the Renewable Transport Fuel Obligation Order (RTFO) (Great Britain Parliament, 2007). The act stipulated mandatory levels for bioethanol content within unleaded petrol sold in the UK – 2.56, 3.89 and 5.26 % for 2008, 2009 and 2010, respectively. Following this, the UK government commissioned an independent assessment of its biofuel policy. The Gallagher review reported to ministers in 2008 and made a number of recommendations. The principal ones being that biofuel implementation should be slowed, that there should be a greater emphasis on 2nd generation feedstocks and that assessment protocols used in assessing green house gas (GHG) savings needed to more accurately account for the indirect effects of land use change. Subsequent to the review the UK government issued the Renewable Transport Fuel Obligations (Amendment) Order (Great Britain Parliament, 2009). The amended act

scaled back the time-scales for implementation of the bioethanol blending specifications outlined by the initial legislation. Updated figures specified bioethanol blending levels of 3.36, 3.62, 4.17, 4.71 and 5.26 % for 2009, 2010, 2011, 2012 and 2013, respectively. Figures for 2012 – 2013 were further confirmed by the Renewable Transport Fuel Obligations (Amendment) Order (Great Britain Parliament, 2011).

Additionally the 2011 amendment order introduced mandatory sustainability criteria which biofuels must meet in order to be contributory to the legislated levels of bioethanol within petrol. Sustainability criteria include minimum green house gas savings alongside obligating fuel suppliers to demonstrate that the feedstocks used in bioethanol production do not damage areas of high carbon stocks or high biodiversity such as forests. As of 2011 UK biofuels targets are being met with biofuels accounting for 4.1% of total liquid transportation fuel usage in the UK (Department for Transport, 2012). However there has been no further legislation implemented to increase biofuel usage past the mandated levels of 5.26 % set for 2013. This is mainly due to the sustainability issues highlighted by EU Council Directive 2009/28/EC with regards to 1st generation bioethanol (discussed in detail later in this chapter). As such it would appear that if bioethanol utilisation in the UK is to continue to increase past 2013 then 2nd generation bioethanol has a critical role to play.

Brazil was something of a pioneer in terms of the development of the world's first large scale integrated bioethanol production network. The use of ethanol as a transportation fuel in Brazil, dates back to the early part of the 19th century, however it was not until the 1930's that ethanol-petroleum

blending took place on a significant scale (Rosillo-Calle and Heatford. 1987). In the 1970's a combination of the energy crisis, coupled with a large scale investment to modernise the sugar industry, led to the introduction of the National Alcohol Programme (PROALCOOL). The aim of the program was simple – to reduce the country's dependence on external imports of crude oil. The first phase of the program, initiated in 1975, targeted the substitution of conventional petroleum products with biomass derived (predominately sugar cane) fuel ethanol. This was achieved through the addition of distilleries into existing sugar production plants, resulting in the production of anhydrous ethanol, which is blended with conventional petroleum products.

1.5 Global Bioethanol production

Global bioethanol production has increased exponentially over recent decades. This increase has seen worldwide production jump from 667.7 million litres in 1975 to 103.4 billion litres in 2011 (Fig 1.3). Global bioethanol production is dominated by the United States and Brazil (Fig. 1.4), who together accounted for ~ 87.8 % of the world's production in 2011 (F.O. Lichts. 2012). In terms of feedstock, 1st generation sources dominate, with the most widely utilised being maize (corn) in the United States and sugar cane in Brazil.

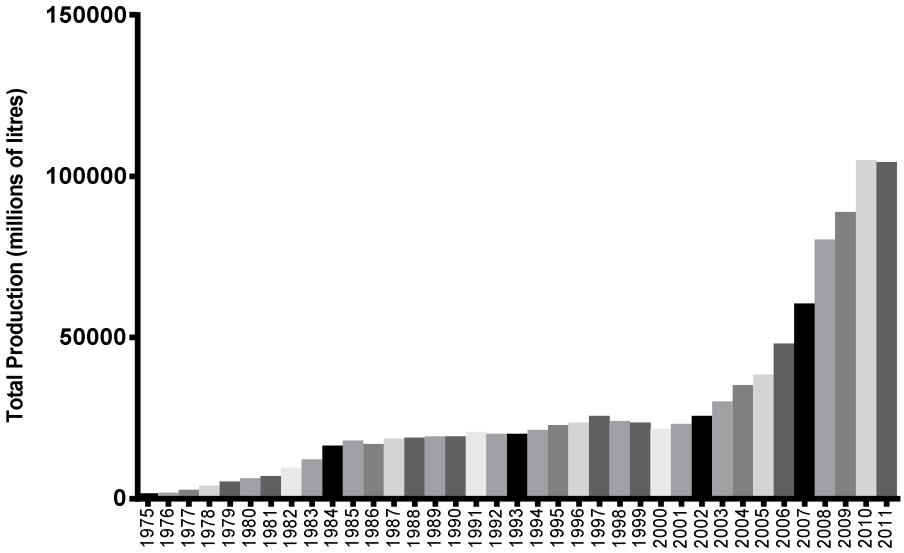
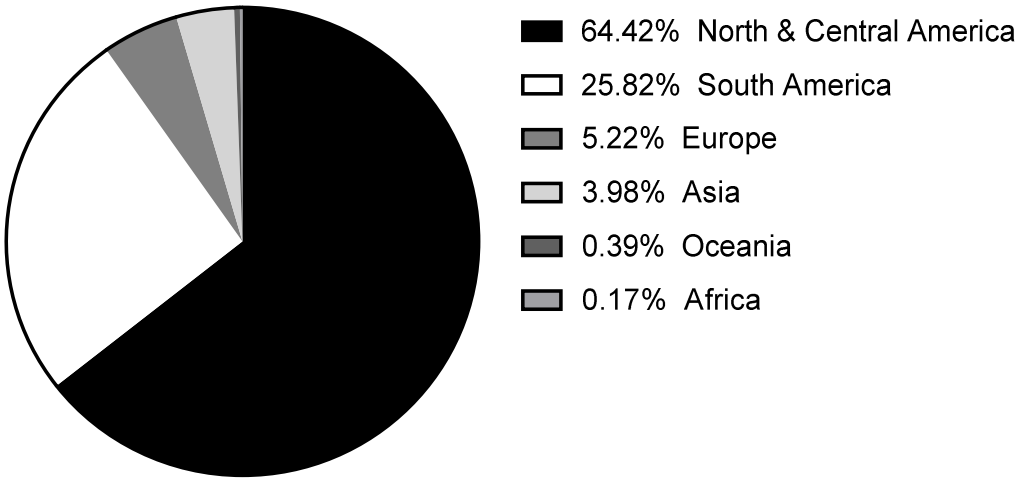


Fig 1.3 – Global bioethanol production from 1975 – 2011



Global total = 103.4 billion litres

Fig 1.4 – Global bioethanol production in 2011 by region

1.5.1 Bioethanol production in the United States

Much of the increase in global bioethanol production can be attributed to increased capacity in the United States, which is now the world's largest bioethanol producer. Production capacity has seen significant growth since 1980 (Fig. 1.5), rising from 795 million litres to 63.19 billion litres in 2011 (F.O. Lichts. 2012). The increase means bioethanol now represents more than 25 % off all domestically produced fuel in the United States. As of January 2012, there were 209 plants producing bioethanol, with a further two under construction, representing an installed capacity of 68.4 billion litres per annum (Renewable Fuels Association, 2012). Most of these are situated in and around the "corn belt" states of Iowa, Nebraska, Illinois, Minnesota and South Dakota to enable easier access to the primary feedstock used in the production of bioethanol, namely, maize.

The United States currently consumes ~ 619.7 billion litres of petroleum per annum, with more than 95 % of this being blended with domestically produced bioethanol, usually in the form of E10 (blend of 10% ethanol and 90% conventional gasoline) (Renewable Fuels Association. 2012). This has led to a reduction in American dependence upon imported petroleum from ~ 60% of its requirements in 2005, to below 50% in 2011.

The market is currently deemed as saturated in terms of supply of E10, a notion commonly referred to as the "blend wall". This idea dictates that once all of the unleaded gasoline sold in the United States contains 10% bioethanol that no further increase in production capacity can be justified as there is a limited market for the excess ethanol. As such, in order for the renewable fuel standard (RFS) to continue to increase bioethanol production,

legislation has been passed that seeks to promote higher blending proportions. This is soon to be realised through the sale of the higher ethanol blend - E15 (blend of 15% ethanol and 85% conventional gasoline).

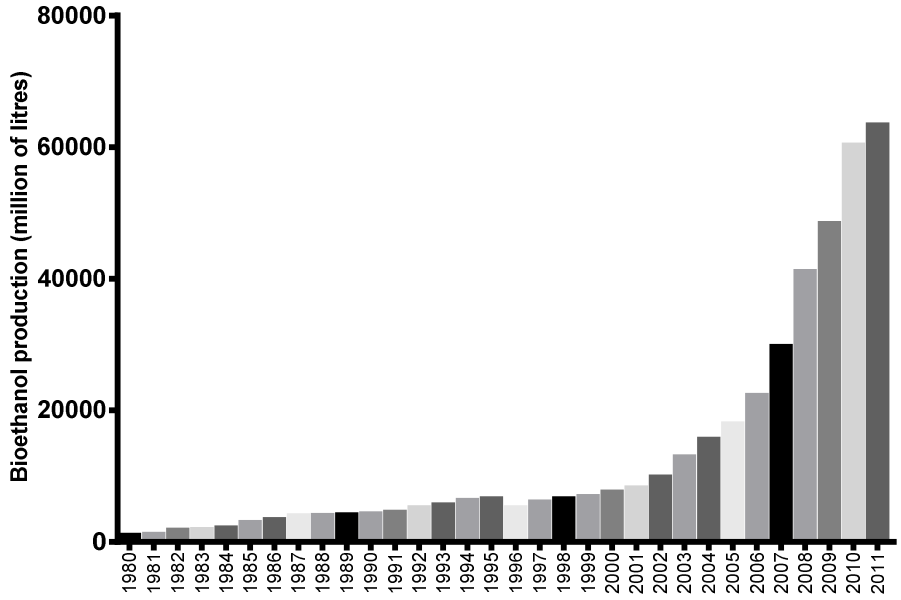


Fig 1.5 – Trends in US bioethanol production 1980 - 2011

1.5.2 Bioethanol production in Brazil

As has been previously discussed, Brazil was the first country to develop an integrated bioethanol production network, with its origins dating as far back as 1970's. As such, compared to other bioethanol producing countries (e.g. the United States), Brazil has seen a lower rate of increase in production capacity during recent years. However, production capacity has been increasing steadily since 1975, rising from 667.7 million litres to 25.2 billion litres in 2011 (Fig. 1.6). Currently there are 448 operational bioethanol production plants in Brazil (Soccol *et al.*, 2010), with a maximum production

capacity of 27 billion litres of bioethanol per annum. This is expected to increase to over 104 billion litres by 2025 (Soccol *et al*., 2010).

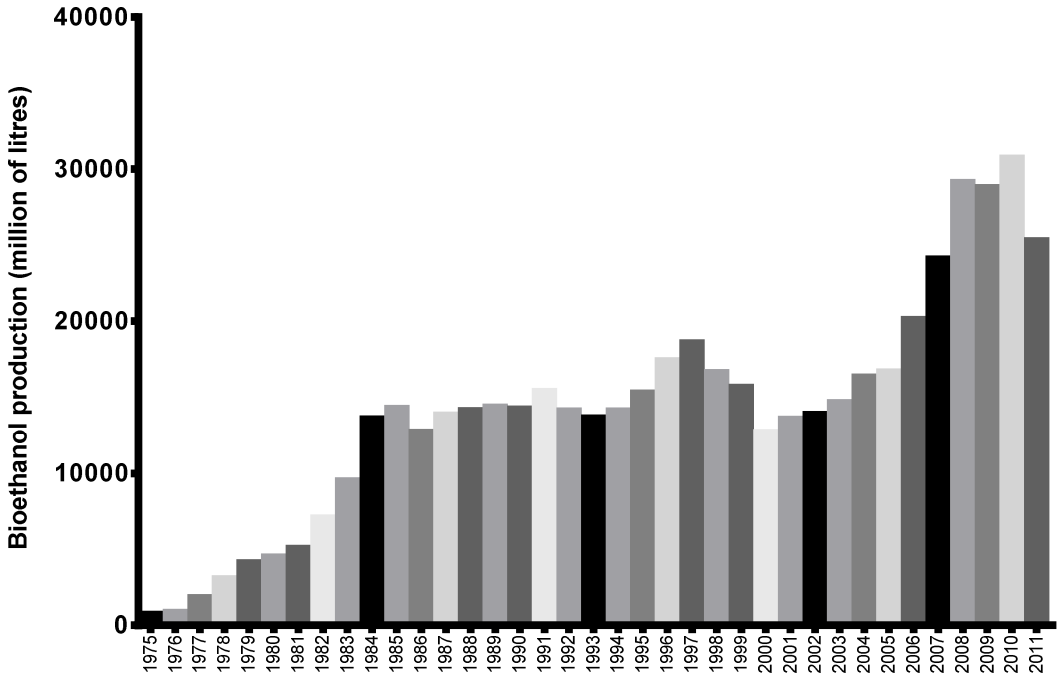


Fig 1.6 – Trends in Brazilian bioethanol production 1980 - 2011

1.5.3 Bioethanol production in the EU

Bioethanol production in the EU is primarily derived from wheat and sugar beet. Arguably, the European Union has been slow on the uptake when it comes to the development of bioethanol production capacity. However, as has been seen with the majority of global bioethanol producing regions, production levels in the EU have increased since 2005 (Fig 1.7). This has seen total production in the region rise from 945 million litres in 2005 to 5.31 billion litres in 2011.

There was however a reduction in bioethanol production year on year between 2010-2011 and this can be attributed EU legislation implemented in 2009 which called for further assessment into “the availability of biofuels made from waste, residues, non-food cellulosic material and lignocellulosic material” (Council Directive 2003/30/EC). As such there is likely limited increase in bioethanol production in EU until 2nd generation feedstocks become mainstream.

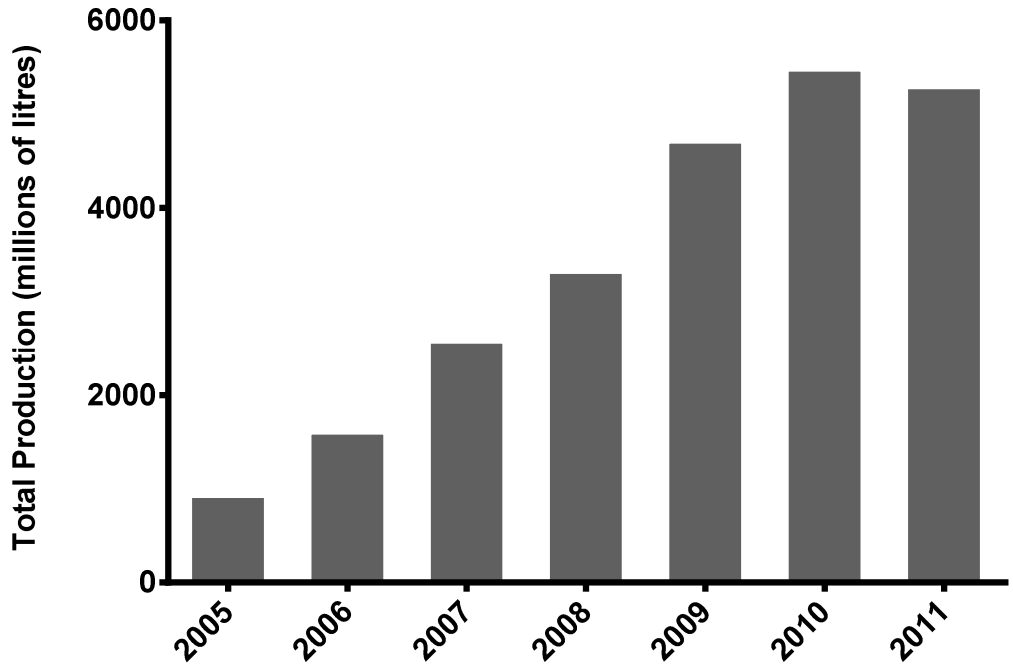


Fig 1.7 EU bioethanol production from 2005 - 2011

1.6 Economics of bioethanol production

The economic and energy balance issues associated with the production of bioethanol are the primary barriers that, until recently, have hindered the mass uptake of bioethanol as a mainstream transportation fuel. Both are

critical in determining the economic feasibility of bioethanol, in terms of providing a realistic alternative to traditional fossil based transportation fuels.

In order to be sustainable bioethanol production must have a positive net energy ratio (NER), that is the ratio between the energy contained in the ethanol and the energy expended in its production must be positive. Without a positive NER, more energy is used during the production of bioethanol than is gained during its combustion, which obviously makes production unsustainable. NER's vary widely between bioethanol derived from different types of feedstock (Table 1.2).The NER's associated with lignocellulose derived bioethanol tend to be higher than those from 1st generation generation feedstocks. However, as is case with 1st generation feedstocks, NER values vary widely between specific types of lignocellulosic biomass. Typically NER values for cellulosic ethanol range from 4 – 35.

The variation in bioethanol NER's can be attributed to a number of factors. It would be logical to assume that 2nd generation ethanol should have a lower NER than 1st generation due to increased energy utilisation during pre-treatment and enzymolysis and the additional energy used by distilling low ethanol yields. However the increased energy expended during production of 2nd generation ethanol is offset by a number of factors.

Variation between 1st and 2nd generation feedstocks is largely due to the fact that 2nd generation feedstocks generally do not have the energy expended during agricultural processes allocated to them (Tan, Lee and Mohamed, 2008). The rationale being, that as they are viewed as waste products the agricultural energy usage is allocated to the primary product (the grain itself), rather than the lignocellulosic waste residue (Fischer *et al*.,

2010). Allocation of all or part of the agricultural energy expended during crop production to the agricultural lignocellulosic residues would ultimately have the effect of reducing the NER's associated with bioethanol derived from waste lignocellulosic residues. If this was to happen there would need to be a re-assessment in terms of the sustainability of 2nd generation fuel ethanol. Coupled with the variation in NER's between 1st and 2nd generation feedstocks, there is also a variation between differing types of 1st generation feedstock. Brazilian 1st generation ethanol has a significantly higher NER than that observed with US corn ethanol. This is largely due to increased efficiency and energy recovery in Brazilian bioethanol distilleries such as energy recovery from the combustion of bagasse (Braunbeck *et al*., 1999).

With the exception of Brazil, bioethanol produced from 1st generation feedstocks tend to have low positive NER's. This is particularly true of corn ethanol in the United States. There is currently a debate raging in terms of whether or not US corn ethanol exhibits a positive energy ratio, however it appears likely that is does, although many studies tend to suggest it is extremely low at around ~ 1.3 (Shapouri *et al*., 2002). Whilst ethanol derived from sugar beet, has an increased NER in comparison with corn – derived ethanol, this increase is marginal with an NER of ~ 2. Brazil is something of an anomaly in terms of NER's of ethanol from 1st generation feedstocks, with a ratio of ~ 7.9, this is by far the highest of any 1st generation feedstock.

Table 1.2 Comparison of bioethanol NER's from various feedstocks (data from Blottnitz and Curran, 2007)

Feedstock and country	NER
Sugarcane, Brazil	7.9
Sugar beet, Great Britain	2
Corn, USA	1.3
Corn stover, USA	5.2
Wheat straw, Great Britain	5.2
Bagasse, India	32
Conventional petroleum	6

1.7 Problems with current production

Current bioethanol production relies heavily on the use of 1st generation feedstocks. This is particularly true of the large biofuel producing regions, with the main sources of fermentable carbohydrate coming from sugar cane, corn, wheat and sugar beet for Brazil, the United States and the European Union respectively. Whilst 1st generation feedstocks provide a means for efficient, technologically simple and large scale production of bioethanol, the situation presents considerable problems in terms of sustainability. This manifests itself both in terms of greenhouse gas (GHG) emissions and the divergence of potential food to fuel.

When considering corn-based ethanol production in the United States, various studies have looked at specific blends of bioethanol in terms of GHG emissions, ranging from E10 through to E85, with contrasting results. Whilst

E10, E20 and E85 are, through the imparted increase in research octane number (RON) rating, slightly more fuel efficient than traditional petrol, the lower energy content of ethanol actually leads to an increase in fuel consumption, thereby increasing CO_2 emissions on a mile for mile basis (Knapp. *et al.,* 1998). When a full life cycle assessment (LCA) of corn based bioethanol is conducted, this increase in CO_2 emissions is offset by the CO_2 sequestration that occurs during corn production i.e. during the photosynthetic activities of maize growth. As such, many studies have concluded that large scale bioethanol production from 1st generation feedstocks in the United States decreases GHG emissions relative to those released from fossil based fuels (Wang *et al*., 1999; Wang, 2000; Kim and Dale, 2005; Farrell *et al.,* 2006). These decreases in GHG emissions vary dependent on the blend of bioethanol that is assessed. For E10 GHG savings of 1-5 % are typical (Wang *et al.,* 1998), for E20 this rises to 2-11 % (MacLean *et al.,* 2000) and for blends in excess of E85 GHG savings lie in the range 19-70 % (MacLean *et al.,* 2000).

However more recent studies have brought to light a number of problems with the research methodology used in earlier work. Searchinger *et al.* (2008) concluded that many earlier studies failed to account for land use change in their assessment of GHG emissions in relation to bioethanol derived from corn. Furthermore, many more recent studies have concluded that corn based ethanol production, rather than being carbon neutral, actually increases greenhouse gas emissions when its LCA is compared with the LCA of conventional transportation fuels. Whichever side of the debate turns out to be correct in relation to the size of GHG savings from 1st generation

corn based bioethanol, if indeed there are any, it is clear that corn derived bioethanol in the United States, is not the panacea it was once believed to be, in terms of its environmental credentials.

Corn derived bioethanol faces additional challenges in terms of sustainability through the so called “food to fuel” debate. This argument centres of the moral issue of diverting a large portion of cereal production in the United States to fuel generation, when more than 800 million people globally are struggling with malnutrition and attempting to feed themselves on less than $1 a day (Banerjee *et al.,* 2007). The United States contributes more than 40 % of all maize produced globally and is responsible for 55 – 60 % of all maize traded globally (Naylor. *et al.,* 2007). As such, both the amount of maize grown in the United States in a given year, as well as the proportion of this used domestically, can have a massive effect upon global grain prices. The “food to fuel” debate in combination with concerns about real world GHG savings lead to the arguable conclusion that current United States 1st generation bioethanol production is unsustainable.

Various studies have assessed the environmental credentials of sugar-cane derived 1st generation bioethanol in Brazil, again with contrasting results. It is generally agreed that Brazilian production of bioethanol from sugar cane represents a significant decrease in GHG emission in comparison to conventional transportation fuels. Reported GHG savings do however vary widely between both studies and levels of bioethanol blending – 87% for E85 (Luo *et al*., 2008), 78% for E25, 70% for E10 (Walter *et al*., 2011). Whilst it appears likely that Brazilian production of bioethanol does represent a significant GHG saving over the use of conventional fuels, there

are growing reports that Brazilian bioethanol is not without its environmental problems. The primary example being the diversion of land once devoted to soya bean production now being used to cultivate sugar cane for bioethanol production (Martinelli and Filoso., 2007).

It is clear that there is a significant debate within the scientific community in terms of the sustainability of 1st generation bioethanol. This is particularly evident in terms of energy balances, the impact of land use change and reductions in GHG emissions. However, regardless of the outcome of this debate, it is abundantly clear that 1st generation bioethanol is not as sustainable as once thought. This in turn suggests that in order for bioethanol to be a realistic and sustainable alternative to conventional fuels, there needs to be a move away from 1st generation feedstocks towards 2nd generation feedstocks obtained from waste materials.

1.8 Second generation bioethanol from lignocellulose

Second generation bioethanol does much to alleviate the environmental concerns associated with 1st generation bioethanol, including circumvention of the "food to fuel" debate and a greatly improved energy ratio. Bioethanol derived from waste sources of lignocellulose represents one of the most attractive, yet challenging, opportunities to develop low-cost and sustainable bioethanol production systems. Abundant sources of waste lignocellulosic biomass come in a variety of forms including: waste residues from the brewing/distilling, forestry and paper industries. Certain forms of domestic waste represent a further avenue for sourcing waste lignocellulose (waste paper etc).

As discussed previously, the production of bioethanol from 2nd generation cellulosic feedstocks is significantly more complex than those of 1st generation feedstocks. The major factors in this regard are that the polymeric carbohydrate material is more difficult to convert to simple fermentable monosaccharides than is the case with starch or sugar based feedstocks. The process stages involved in the production of bioethanol from lignocellulosic substrates are detailed in Fig. 1.8.

1.8.1 Spent grains

Brewer's spent grains (BSG) and distiller's spent grains (DSG) are the residual cereal grain material remaining subsequent to starch hydrolysis and extraction in the brewing and distilling industries, respectively. They form a readily available source of waste lignocellulose, which has previously been shown to be a rich source of fermentable carbohydrate (White *et al*,. 2008). This type of waste from the brewing and distilling industry is currently sold as animal feed, and systems which could generate value added products from it are of current interest.

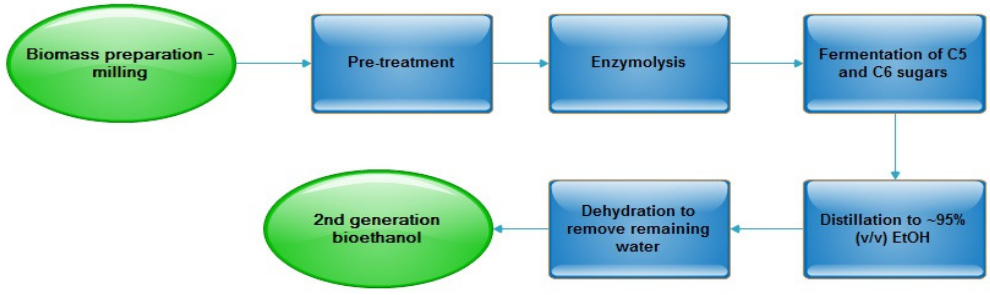


Fig.1.8 Process steps in the production of bioethanol from lignocelluloses

1.8.2 Composition and structure of lignocellulose

Lignocellulose is a matrix comprising cellulose, hemicellulose, and lignin. Cellulose is a polysaccharide of glucose subunits joined by β-1, 4 glycosidic bonds, which comprises large sections which are crystalline in nature (Fan *et al*, 1982). Hemicellulose is a branched heteropolysaccharide consisting of various co-polymers: the pentoses D-xylose and L-arabinose, and the hexoses D-glucose, D-mannose and D-galactose (Saka, 1991). Additionally, hemicellulose is heavily acetylated, with high levels of acetyl functional groups found along its side chains. However, the exact composition of hemicellulose varies greatly between differing types of biomass. Lignin is a complex hydrophobic aromatic polymer containing phenylpropanoid monomers, principally p-coumaryl alcohol, conferyl alcohol and sinapyl alcohol. Lignocellulose is one of the most abundant substances on earth, forming the structural support material in the majority of plants and woods.

The components within lignocellulose come together to form a complex structure (Fig. 1.9) which is found in the plant cell wall. The cellulose chains are bound to each other through hydrogen bonding which packs them together into a structure termed a micro-fibril. Hemicellulose surrounds the microfibril through covalent linkages to the cellulose chain. Adjoining fibrils are bonded to each other by lignin and other polymers such as pectin which are bonded to the hemicellulose. This creates a bundle of tightly packed micro-fibrils which is referred to as a macro-fibril. Due to the fact that lignin occupies the outer of region of the micro-fibril and surrounds the cellulose and hemicellulose chains it plays a number of roles, the primary one being to provide structural strength to the macro-fibril and protect the

polysaccharide component from external attack. Evolution has designed the structure of lignocellulose to be resistant to degradation and lignin plays a key role in protecting plants and trees from biological (e.g. fungal attack) and environmental elements (e.g. wind and rain).

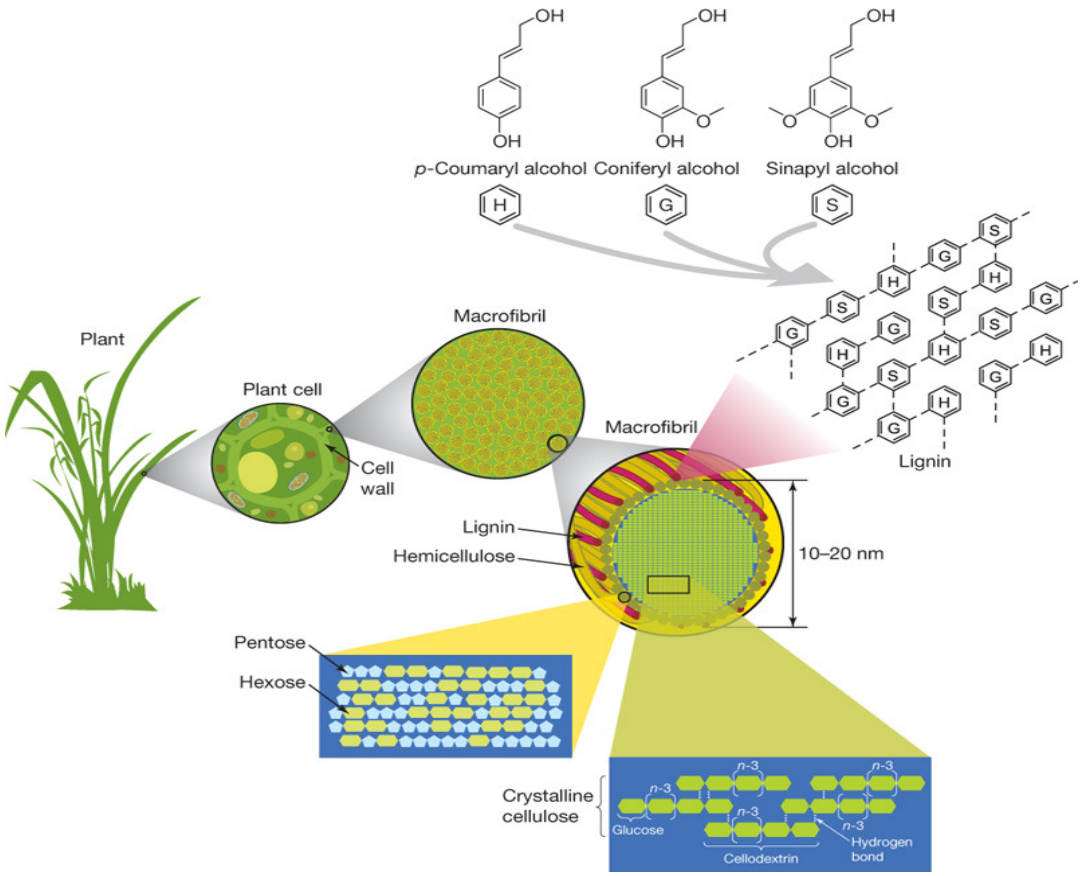


Fig. 1.9 Structure of lignocellulose

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Lignocellulose is a generic term that is used to classify any plant structural support matrix. It varies greatly in composition between plant species (Table 1.3), with varying amounts of cellulose, hemi-cellulose and lignin. Whilst the composition of lignocellulose varies greatly between different types of biomass, spent grains typically consist of ~ 17% cellulose, ~ 28% non-cellulose polysaccharides (hemicellulose, mainly arbinoxylan)

and 28% lignin (Mussato *et al.*, 2006). As such spent grains compare favourably with other forms of lignocellulosic biomass for conversion to bioethanol in that they contain similar levels of extractable carbohydrate for subsequent fermentation. However they contain relatively high levels of lignin compared to other biomass types such as barley straw (Table 1.3). Higher lignin content could render them more difficult to pre-treat than other biomass types.

Table 1.3 Variation in biomass composition

	Component composition (% dry w/w)			
Feedstock	**Cellulose**	**Hemi-cellulose**	**Lignin**	**Reference**
Barley hull	34	36	19	Kim, Taylor and Hicks, 2008.
Barley straw	36 - 43	24-33	6.9 - 9.8	Garda-Aparicio *et al.,* 2006.
Bamboo	49 - 50	18 - 20	23	Alves *et al.,* 2010.
Corn cob	32.3 - 45.6	39.8	6.3 - 17.9	Cao *et al.,* 1997, McKendry, 2002.
Corn stover	35.1 - 39.5	20.7 - 24.6	11 - 19.1	Mosier *et al.,* 2005.
Cotton stalk	31	11	30	Rubio *et al.,* 1998.
Douglas fir	35 - 48	20 - 22	15 - 21	Schell, Ruth and Tucker, 1999.
Eucalyptus wood	45 - 51	11 - 18	29	Alves *et al.,* 2010, Pereira, 1988.
Rice straw	29.2 - 34.7	23 - 25.9	17 - 19	Brylev *et al.,* 2001, Prassad, Singh and Joshi, 2007.
Rice husk	28.7 - 35.6	11.96 - 29.3	15.4 - 20	Allen *et al.,* 2001, Abbas and Ansumali, 2010.
Wheat straw	35 - 39	22 - 30	12 - 16	Prassad *et al.,* 2007, Grohmann, Torget and Himmel, 1985.
Wheat bran	10.5 - 14.8	35.5 - 39.2	8.3 - 12.5	Miron, Yosef and Ben-Ghedalia, 2001.
Sugarcane bagasse	25 - 45	28 - 32	15 - 25	Alves *et al.,* 2010, Singh *et al.,* 2009.
Pine	42 - 49	13 - 25	23 - 29	Rubio *et al.,* 1998.
Poplar wood	45 - 51	25 - 28	10 - 21	Pereira, 1988.
Switchgrass	35 - 40	25 - 30	15 - 20	Howard *et al.,* 2003
Sorghum straw	32 - 35	24 - 27	15 - 21	Herrera *et al.,* 2003, Vazquez *et al.,* 2007

1.8.3 Pre-treatment of lignocellulose

The structure of lignocellulose dictates that in order to extract fermentable carbohydrate from the biomass, it must first be pre-treated to render it susceptible to enzymatic hydrolysis. Lignin characterises lignocellulose as being extremely resistant to hydrolysis and sugar extraction. The pre-treatment degrades lignin bound to the cellulose fraction (Mosier *et al.*, 2005) and partially hydrolyses hemicellulose resulting in the release of pentose sugars, chiefly xylose and arabinose. This results in increasing cellulose accessibility, thus rendering it susceptible to enzyme attack. In addition to this, pre-treatment decreases cellulose crystallinity thereby increasing levels of amorphous cellulose.

Whilst cellulose is similar to starch in that they are both polymers of glucose, cellulose has a more crystalline structure (Parisi, 1989). The structure of cellulose favours ordering of the polymer chains into tightly packed crystalline structures that render it water insoluble and resistant to hydrolysis (Mosier *et al.*, 2005). However regions of the cellulose fibre are non-crystalline in nature with larger spaces between individual cellulose chains within the micro-fibril. These particular regions are defined as amorphous cellulose and are more sensitive to enzymatic digestion than crystalline regions. (Fan *et al.*, 1982; Parisi, 1989)

Current approaches to extracting fermentable carbohydrate from lignocellulosic biomass can be separated into four categories: physical, physico-chemical, chemical or biological (Sun and Cheng, 2002; Menon and Rao, 2012; Balat, 2010)

1.8.3.1 Physical pre-treatment

Physical pre-treatment usually involves some form of mechanical processing (e.g. milling) utilised to reduce biomass particle size. In reality physical treatment is rarely used alone as most lignocellulosic biomass is usually milled before being further processed using chemical or physico-chemical methods. In saying that however, certain types of physical treatment exist that can be used as a stand-alone pre-treatment method. These usually involve some form of biomass irradiation typically with gamma rays, electron beam or microwave radiation (Menon and Rao, 2012). One type of physical irradiation that is coming under increasing focus is ultrasound, the use of which will be discussed in further detail later in this chapter.

1.8.3.2 Physico-chemical pre-treatment

Physico-chemical pre-treatment is a term that is used to refer to pre-treatments that combine both chemical and physical techniques. Various forms of this type of pre-treatment exist, including; steam, steam explosion, catalysed steam explosion, ammonia fibre explosion (AFEX), liquid hot water and microwave chemical pre-treatment.

Steam explosion, as the name suggests, refers to the use of super-heated steam in the pre-treatment of lignocellulose (Varga *et al.*, 2004). Typically, the biomass is treated with high-pressure steam with parameters of 160 – 260 ℃ and a pressure of 0.5 – 5 MPa. These parameters are usually held for a residence time of ~10 mins, at which point the pressure is dramatically reduced, causing the biomass to undergo explosive decompression. The process results in hemi-cellulose hydrolysis and lignin

degradation, thereby increasing the susceptibility of the cellulose fraction to further hydrolysis enzymatically. Steam explosion has been reported as an effective pre-treatment mechanism for a variety of biomass types (Ruiz *et al*., 2006, Kurabi *et al*., 2005).

AFEX is a pre-treatment method which is similar to steam explosion, however instead of water, the biomass is pre-treated with ammonia. A typical methodology for this type of treatment involves dosing liquid ammonia at ~ 1 kg ammonia per kg of biomass and holding it at a temperature of ~ 90 ℃ for 30 mins. The process causes partial degradation of lignin and hemicellulose and decreases cellulose crystallinity, thereby increasing the susceptibility of the biomass to enzymatic degradation. AFEX has been used to successfully pre-treat lignocellulosic biomass in a number of instances (Gollapalli *et al*., 2002; Teymouri *et al*., 2004).

Liquid hot water (LHW) involves the use of hot water in the pre-treatment of lignocellulose. In a typical setup this is performed at pressure to stop water being gaseous. In essence, the process involves the high temperature cooking of the biomass in water. LHW has been shown to successfully pre-treat straw (Yu *et al*., 2010; Ingram *et al*., 2009; Pérez *et al*., 2007) and poplar (Kim *et al*., 2009).

Microwave pre-treatment of lignocelluloses involves the use of microwave radiation during the pre-treatment process and is usually combined with some form of chemical treatment. As this is a new and emerging technology reports as to its effectiveness are limited, however the studies that have been reported, suggest that it is a viable form of

lignocellulose pre-treatment technology. Microwave radiation in combination with alkaline pre-treatment has been reported as more effective than alkaline treatment alone (Zhu *et al*., 2005). The same has been reported of microwave radiation in combination with treatment with acid or hydrogen peroxide (Zhu *et al*., 2006).

1.8.3.3 Chemical pre-treatment

Chemical pre-treatments are the most widely researched pre-treatment method for lignocellulose. They were originally developed by the paper industry for the delignification of woody biomass in order to produce paper products. There are five main types of chemical pre-treatment these being; acid, alkaline, ionic liquids, ozonolysis and treatment with hydrogen peroxide.

Acidic pre-treatment refers to the use of dilute acid in the pre-treatment of lignocellulose. The most commonly utilised acids are H_2SO_4, HCl and to a lesser extent HNO_3 with the protons (H^+) in the acid being the mechanism by which lignin and hemicellulose are oxidised. This results in degradation of lignin and hemicellulose, and a decrease in cellulose crystallinity thereby rendering it susceptible to enzymatic attack. H_2SO_4, HCl and HNO_3 have all been reported to be effective in the pre- treatment of a wide variety of lignocellulosic biomass, including; spent grains (White *et al*., 2008), corn stover (Du *et al*., 2010), switchgrass (Digman *et al*., 2010; Li *et al*., 2010), spruce (Shuai *et al*., 2010), poplar (Wyman *et al.,* 2009), eucalyptus wood (Romani *et al*, 2010) and wheat straw (Deprez *et al*., 2009). A typical methodology for the use of acid in lignocellulose pre-treatment involves adding acid to the biomass at a concentration between 0.1-1.5 M,

followed by heat treatment between 120 – 210 ℃, with a residence time of up to 3 hours. The efficiency of the pre-treatment is highly dependent upon the process parameters utilised during pre-treatment. Acid concentration, temperature and residence time all play a role in this regard (Sun and Cheng, 2005; Saha *et al*., 2005; Xiang *et al*., 2003).

Alkaline pre-treatment relates to the use of basic solutions, such as sodium, potassium and ammonium hydroxide, as well as lime, in the pre-treatment of lignocellulosic biomass. It has been reported as being effective in pre-treating a wide variety of feedstocks, including straw (Ibrahim *et al*., 2011), grass (Sills and Gossett, 2011), corn stover (MacDonald *et al*., 1983) and sunflower hulls (Soto *et al*., 1994). Pre-treatment with alkaline solutions is believed to result in the saponification of intermolecular ester bonds that cross-link between lignin and hemicellulose (Sun and Cheng, 2002). This results in lignin removal from the lignocellulose matrix, partial solubilisation of hemicellulose and a partial decrease in cellulose crystallinity (Cheng *et al*., 2010, McIntosh and Vancov, 2010). Process steps in the alkaline pre-treatment of lignocelluloses are closely aligned with those utilised during acidic pre-treatment, with differences in temperature and treatment residence time. The process involves mixing the biomass with a basic solution (usually NaOH) and incubating for an extended period of time at 20℃.

The use of ionic liquids in the pre-treatment of lignocellulose, are a recent development within the field of cellulosic ethanol. It involves the use of so called designer solvents that are liquid at ambient temperature and consist entirely of ionic chemical species (Earle and Sneddon, 2000). The physical of attributes of ionic liquids lead to a reduced lattice energy of the

crystalline form of the salt and thereby infer a lower melting point. To date, two types of ionic liquids exist – simple salts consisting of a single anion and cation, and binary ionic liquids. Examples of ionic liquids, include; 1-butyl-3-methylimidazolium chloride (BMIMCl) (Dadi *et al*., 2006), 1, 3-N-methylmorpholine- N-oxide (NMMO) (Kuo and Lee, 2009) and 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl) (Binders and Raines, 2010), which were reported as successful in the pre-treatment of switchgrass, bagasse and corn stover, respectively.

The use of ozone in the pre-treatment of lignocellulose is a new and emerging pre-treatment technology that is gaining increasing prevalence in peer reviewed publications. Ozone is highly oxidising and is extremely reactive with specificity towards compounds with double bonds and functional chemical groups containing high electron densities (García-Cubero *et al*., 2009). Thus, when lignocellulose is ozonolysed the most likely component to be oxidised is lignin, due to its high number of carbon – carbon double bonds. Ozone is highly effective in oxidising and degrading lignin. This results in the removal of lignin from the lignocellulose structural matrix and renders cellulose and hemicellulose susceptible to enzymatic hydrolysis. Ozone has been reported to be efficient in the pre-treatment of various form of lignocellulosic biomass, including; corn stalks (Quesada *et al*., 1999), cotton stalks (Silverstein *et al*., 2007), wheat and rye straw (García-Cubero *et al*., 2009) and poplar sawdust (Vidal and Molinier, 1988).

Hydrogen peroxide has been shown to be effective in the pre-treatment of lignocellulose through oxidative de-lignification and reduced cellulose crystallinity (Gould, 1985). Its mechanism of action is similar to that

of ozone, in that oxidisation of lignin results in the removal of lignin from the microfibril which renders cellulose and hemicellulose accessible to hydrolytic enzymes. Hydrogen peroxide has been reported to be effective in the de-lignification of a variety of lignocellulose feedstocks, including; wheat straw (Martel and Gould, 1990), oak (Kim *et al*., 2001) and Douglas fir (Yang *et al*., 2002). A typical methodology for the use of hydrogen peroxide in the pre-treatment of lignocellulose involves mixing the biomass with ~ 1 % H_2O_2 and incubating it at an ambient temperature for 24 – 48 h. Following this the biomass is rendered amenable to enzymatic extraction of the biomass sugars.

1.8.3.4 Biological pre-treatment

Biological pre-treatment normally involves the use of various wood decay fungi, usually white, brown or soft rot fungi which possess the ability to break down lignocellulose and increase its susceptibility to enzymatic hydrolysis. White rot fungi are often reported as the most effective for the pre-treatment of lignocellulose and are often the basis for fungal pre-treatment due to being the most effective in lignin degradation through their unique ligninolytic systems (Eriksson et al., 1990; Wan and Li, 2012).

Several species of white rot fungi have been shown to be effective in pre-treating a variety of biomass types, including: *Phaerochaete chrysosporium* pre-treatment of cotton stalk (Shi *et al*., 2009), corn stover (Keller *et al*., 2003) and rice straw (Bak *et al*., 2009) and *Pleurotus ostreatus* pre-treatment of rice straw (Taniguchi *et al*., 2005) and wheat straw (Hatakka, 1983). White rot fungi utilise a number of mechanisms to degrade

lignocellulosic biomass and this usually involves a combination of hydroxyl radicals, lignin peroxidise and laccase enzymes to degrade lignin and hydrolytic enzymes such as cellulase to degrade the polysaccharide component of lignocellulose. (Eriksson et al., 1990; Wan and Li, 2012)

Fungal pre-treatment is often used prior to the use of a further physical/ chemical pre-treatment. This has been shown to increase the effectiveness of a number of more conventional chemical pre-treatments such as alkaline (Hatakka, 1983) and dilute acid (Ma *et al*., 2010). However the use of fungal pre-treatment is severely limited within an industrial context as the incubation time required is typically long ranging from days to weeks depending on feedstock (Wan and Li, 2012).

1.8.3.5 Limitations of current pre-treatment technologies

Whilst all of the pre-treatment technologies discussed so far, possess, to varying degrees, the ability to render lignocellulosic biomass susceptible to enzymatic digestion, most of them present considerable disadvantages of one form or another.

Pre-treatment is a trade-off between efficient enzymolysis and fermentation. Whilst pre-treatment is required to enable efficient enzymolysis, the high temperatures usually employed in its application generate a number of chemical species which are inhibitory to yeast fermentation. For example, hemicellulose (arabinoxylan) hydrolysis yields acetic acid, and pentose and hexose sugars released during cellulose/hemicellulose hydrolysis can be further degraded to furfural and hydroxymethyl furfural (HMF), respectively. Both of these compounds can be

further degraded to formic and levulinic acid (Palmqvist and Hahn – Hagerdal, 1999). Lignin degradation products comprise a variety of phenolic compounds, the majority of which are inhibitory to yeast fermentation. A number of methods exist which have the potential to detoxify lignocellulosic hydrolysates including: overliming, filtration through activated charcoal and liquid-liquid extraction utilising an organic solvent.

As has been outlined, a myriad of lignocellulose pre-treatment technologies have been extensively studied over a prolonged period of time. Whilst many are effective in the pre-treatment process, none represent an ideal technology, with all of them displaying various advantages and disadvantages. Various parameters must be taken into account when evaluating the applicability of a pre-treatment technique to a given biomass type. An effective pre-treatment method is characterised by; being able to effectively degrade lignin, preserving hemicellulose thereby limiting fermentation inhibitor formation, decreasing cellulose crystallinity, minimizing energy input and being cost effective. A pre-treatment that satisfies all of these criteria will render both cellulose and hemi-cellulose amenable to hydrolysis enzymatically and ultimately result in a sugar rich, highly fermentable hydrolysate containing low levels of inhibitors. Unfortunately, to date, it is debatable as to whether or not such a pre-treatment technology exists that satisfies all of these criteria.

1.8.4 Enzymatic digestion of pre-treated lignocellulose

Many of the barriers to cellulosic bioethanol centre on the cost, efficiency and energy balance of the process techniques used in its production. In the past,

cellulolytic enzymes have proved prohibitively expensive, causing the economies of production to be so adverse, that the commercialisation of the technology remained unviable. However, recent developments in enzyme production techniques have improved this. Cellulolytic enzymes are typically derived from fungal species such as *Trichoderma ressei*, *Neurospora crassa* or *Fusarium oxysporum*. However the enzyme yields obtained from using these fungi are often unsatisfactory for the commercial production of cellulase (Mathew *et al*., 2008). Consequently, much of recent enzyme research has focused upon increasing enzyme yields though the utilisation of GM fungi and bacteria (Mathew *et al*., 2008).

Following pre-treatment, lignocellulose is incubated with hydrolytic enzymes, typically a mix of cellulase, xylanase and β – glucosidase. Cellulase enzymes form a major component of the range of enzymes that are critical in the enzymolysis of pre-treated lignocelluloses. They are produced by a number of organisms including fungi, bacteria and plants, although in the case of lignocellulose cellulolysis, the enzymes are usually obtained from various species of fungi, typically *Trichoderma ressei*, *Neurospora crassa* or *Fusarium oxysporum*. Cellulase enzymes fall under two main classes; exocellulase and endocellulase. Endocellulase (endoglucanase or 1,4-β-D-glucan- 4- glucanohydrolyase (EC 3.2.1.4) acts to cleave internal glycosidic bonds at random points along the cellulose chain, thus exposing individual chains to further hydrolysis (Henrissat *et al*., 1998) Exocellulase (EC 3.2.1.91), 1,4- β -D-glucan cellobiohydrolase or simply cellobiohydrolase (CBH) comprises two enzymes - CBHI and CBHII, which cleave cellobiose subunits from the reducing and non-reducing ends of

the cellulose chain, respectively (Bommarius *et al*.,2008). Cellulase suffers from end-product inhibition, in that high concentrations of cellobiose can inhibit its function. Subsequent to cellobiose release, β – glucosidase hydrolyses the disaccharide to free glucose. Xylanase hydrolyses residual hemicellulose, not degraded during pre-treatment, to glucose, arabinose, xylose and low levels of galactose and mannose.

1.8.5 Fermentation of sugars derived through lignocellulose hydrolysis

Following pre-treatment and enzymatic digestion, the residual biomass (mainly comprising klason lignin and ash) is separated from the liquid component, resulting in a lignocellulose hydrolysate which contains high levels of fermentable carbohydrate. The range and concentration of the component monosaccharides vary dependent on biomass type. In the case of spent grains, the hydrolysate typically contains high levels of glucose, arabinose and xylose, with little or no, mannose and galactose (White *et al*., 2008). The sugars in the resultant hydrolysate are converted to ethanol via an anaerobic yeast fermentation, during which the yeast metabolises the sugars created during biomass pre-treatment and enzymolysis. Standard laboratory strains of *S.cerevisiae*, along with strains utilised by the alcoholic beverage industry, can ferment the major sugar within biomass hydrolysates (glucose). This also applies to the minor component hexose sugars galactose and mannose. However, they lack the capability to metabolise the pentose sugars (arabinose and xylose) found within lignocellulose derived hydrolysates.

Glucose fermentation proceeds via a complicated catabolic pathway (Fig. 1.10). The molecule is transported into the yeast cell by the cell wall membrane hexose transporter (HXT). Following uptake, glucose catabolism proceeds via the Embden – Meyerhof glycolytic pathway (glycolysis). The pathway leads to the oxidation of glucose to pyruvate, resulting in the synthesis of two molecules of ATP per glucose molecule catabolised. Pyruvate is then converted to acetaldehyde by pyyruvate decarboxylase resulting in the formation of two molecules of CO_2. Acetaldehyde is subsequently converted to ethanol by alcohol dehydogenase.

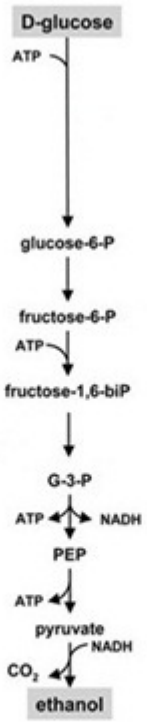


Fig. 1.10 Hexose catabolism in fermenting yeasts (adapted from van Maris *et al.*, 2006)

Various strains of wild-type yeast species possess the capability to ferment the both the hexose and pentose sugars present with lignocellulose hydrolysates, these include; *Pichia Stipitis, Kluyveromyces marxianus, Candida shehatae and Pachysolen tannophilus.* Following pentose uptake into the cell both xylose and arabinose are converted to D-xylulose-5-phosphate though a series of catabolic reactions (Fig. 1.11). D-xylulose-5-phosphate then feeds into pentose phosphate pathway, a pathway which results in the formation of glyceraldehyde 3-phosphate and fructose-6-phosphate. These products then feed into the glycolytic pathway for conversion to pyruvate, which is subsequently converted to ethanol.

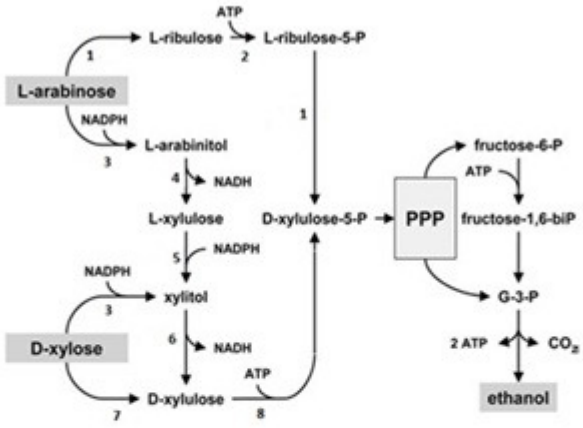


Fig. 1.11 Pentose catabolism in fermenting yeasts (adapted from van Maris *et al.*, 2006)
Numbering indicates enzyme responsible:
1 - L- arabinose isomerase, 2 - L - ribulokinase, 3 - aldose/xylose reductase,
4 - arbinitol 4-dehydrogenase, 5 - L- xylulose reductase, 6 - xylitol dehydrogenase,
7 - xylose isomerase, 8 - xylulokinase.

Fermentations of lignocellulose hydrolysates are typically characterised by slow rates of sugar uptake alongside poor ethanol yields. For this reason, much of the yeast research that is being conducted with regards to the fermentation of sugars derived from lignocellulose, is centred on producing novel GM yeasts. These yeasts, more often than not, comprise a genetically modified strain of *Saccharomyces cerevisiae* that has been engineered to ferment pentose sugars (Bettiga *et al*., 2008), through the insertion of genes from pentose utilising yeasts into the genome.

A variety of fermentation configurations have been developed in order to ferment lignocelluloses hydrolysates including, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated biomass processing (CBP). SHF is the conventional method for the fermentation of sugars derived from lignocellulosic biomass. It is a three step batch process where the lignocellulose is pre-treated in the first step, further hydrolysed enzymatically in the second step and the sugars released are then fermented in the third step. The advantages of this process are that each step can be processed at the optimum conditions required for each stage.

SSF involves the enzymatic saccharification of lignocellulose alongside the fermentation in a single step, dependent on the biomass type this may or may not include a prior pre-treatment stage. Selecting parameters for pH, temperature and substrate concentration is of critical importance to the success of the SSF process (Ballesteros *et al*., 2004). One of the main advantages of this process is that as the sugars are hydrolysed

from the biomass, they are immediately taken up by the yeast and fermented. This results in a faster rate of enzymatic hydrolysis, as it removes the phenomenon of end product inhibition described earlier in this chapter. However the process has a number on disadvantages. The main one being that the yeasts used in the fermentation must be thermotolerant, to allow the process to be conducted at the optimum temperature for the enzymes. This reduces the number or yeasts that are suitable candidates to be used in the fermentation.

Consolidated bioprocessing (CBP) is an emerging technology that is similar to SSF, but differs in a number of ways. In conventional SSF the enzymes used in the digestion of lignocellulose are added to the process externally. Whilst in a CBP system the enzymes are actually produced and released by the fermenting micro-organism. Theoretically, CBP could be used to increase the economic feasibility of cellulosic ethanol by removing the costs associated with the production of hydrolytic enzymes. CPB require the development of a microbe that combines both production of cellulolytic enzymes that enable rapid rates of hydrolysis alongside a high degree of sugar utilisation with fast ethanol formation (Menon and Rao. 2012). However, to date no such organism has been developed.

1.9 Ultrasound

Ultrasound is defined as a cyclic sound pressure wave with a frequency greater than the upper limit of human hearing (usually in the region of 18 – 20 kHz). Ultrasound has a number of uses industrially however two of the

most well-known are medical sonography (Ward *et al*., 1997) and maritime sonar systems (Bom *et al*., 1972).

1.9.1 The origins of ultrasonics

The field of ultrasonics can trace its origins back to the 1940's when it was investigated as a means of tracking submarines. Between the time of its first implementation as a useful tool and the present, many new uses for this technology have emerged including medical diagnostic sonography, industrial ultrasonics, sonochemistry and a variety of biotechnological applications such as acceleration of bioprocesses.

Sonochemistry is defined as the study of the effects of ultrasonic waves upon chemical systems. The field can trace its origins to 1927 with a publication entitled "The chemical effects of high frequency sound waves: A preliminary survey" (Woods and Loomis, 1927). However it was not the 1950's and 1960's that research into this emerging field began in earnest. In those early years work consisted mainly of crude experiments without any real understanding of the phenomena involved. Initial experimentation during this era ultimately deduced the real mechanism by which ultrasound exerts itself upon its target within a liquid medium, namely acoustic cavitation. It was found there are two main types of cavitation; transient and stable with both types able to be produced by the actions of an acoustic field within a gaseous or liquid medium (Crum and Reynolds, 1985).

Transient cavitation refers to bubbles within the medium which undergo irregular oscillations induced by the actions of a sonic wave upon the medium. Due to oscillating irregularly the bubble is destined to implode creating localised increases in temperature and pressure and the ejection of a high pressure jet released from the imploding bubble boundary.

Stable cavitation refers to the phenomenon in which micro bubbles within a medium under the influence of an ultrasonic acoustic field oscillate in a regular fashion. In contrast to transient cavitation, this type of cavitation results in oscillation of the bubbles for extended acoustic cycles, a characteristic which gives rise to micro-streaming. It was not until the 1980's that major advances within this field were to be realised. Research began investigating the best way to utilise this emerging technology as a useful tool, mainly centring on probing the effects of sonic waves on organic and inorganic synthesis

1.9.2 The use of ultrasound in biotechnology

The effects of sonic waves upon chemical systems (sonochemistry) are an increasingly well understood phenomenon, however the same cannot be said of biological systems, with little information existing in the primary literature. However, this situation is starting to change and there are a number of reports detailing the effects of ultrasonic irradiation within a context of biotechnology and bioprocess technology (Kwiatkowska *et al*., 2011)

Ultrasound has the potential to stimulate enzyme function, however this potential is closely linked to the output parameters of the ultrasonic field

(frequency, power (wattage) and temperature), with high power ultrasound tending to denature the structure of the enzyme. Several papers have reported that high power ultrasound causes a decrease in enzyme activity, however this has been found to be the case mainly with cavitating ultrasound (Dunn and Macleod, 1968).

Ultrasound has been shown to increase lipase activity at 30 ° C, however enzyme denaturation was observed when ultrasound was applied at 50 ℃ (Goodman and Dugan Jr., 1970). The activities of invertase (Sakakibara, *et al*., 1996) and pectinase (Yachmenev *et al*., 2001) have been shown to be increased by exposure to ultrasonic irradiation. Several studies have reported the exposure to ultrasound increases the activities of the enzymes involved in starch hydrolysis; alpha-amylase (Schmidt *et al*., 1987; Apar *et al*., 2006; Barton *et al*., 1996), glucoamylase (Schmidt *et al*., 1987) and amyloglucosidase (Barton *et al*., 1996). Other studies have reported that ultrasound increase the efficiency of enzymatic digestion of cotton (Yachmenev *et al*., 2004). In terms of lignocellulose enzymolysis, few studies exist which examine the effects of ultrasonic irradiation upon the enzymes employed during lignocellulose degradation. However, the studies that do exist suggest that ultrasound has the potential to increase sugar release during enzymolysis of cellulose with cellulase. (Yachmenev *et al*., 2009; Condon *et al*., 2009; Yasuda *et al*., 2010; Yoshimoto *et al*., 2004; Aliyu and Hepher, 2000; Wang *et al*., 2012).

The mechanism by which ultrasound enhances the function of hydrolytic enzymes is currently unknown. However there are a number of mechanisms that have been theorised. These mechanisms centre up the

proposal that ultrasound increases the rate at which enzyme proteins interact with their target substrate. Postulated mechanisms include that ultrasound decreases the liquid solid diffusion layer, enhances emulsification, generates micro-streaming, alters surface potential or accelerates molecule transport (Kwiatkowska *et al*., 2011) and these are discussed in depth in Chapter 6.

1.9.3 The use of ultrasound in the pre-treatment of lignocellulose

The use of ultrasound in the pre-treatment of lignocellulose is a research area that is gaining increasing interest. A limited number of studies exist that assess the use of solely ultrasonic irradiation as pre-treatment technique, with the majority of research looking to ultrasound as a means to enhance conventional chemical pre-treatment methods.

Ultrasound has been shown to enhance a variety of chemical lignocellulose pre-treatment techniques. It has been shown to be effective in increasing the efficiency of alkaline extraction of pentose sugars from the xylan fraction of corn cob (Hromadkova and Ebringerova, 1998). The same researchers later reported that the application of ultrasound increased the efficiency of alkaline extraction of hemicellulose pentose sugars from buckwheat hulls (Hromadkova and Ebringerova, 2003) and wheat bran (Hromadkova and Ebringerova, 2008). Ultrasound has also been reported as effective in increasing the efficiency of both a combined alkaline and ionic liquid pre-treatment of poplar wood (Yuan *et al*., 2010), alkaline pre-treatment of wheat straw (Sun *et al*., 2002) and combined lignin extraction from bamboo with ultrasound and ethanol (Li *et al*., 2012).

Other areas look to assess the effects of ultrasound upon degradation of cellulose from de-lignified lignocellulose. Ultrasound has been used in the degradation of both cellulose powder and was found to be effective in reducing particle size in micro-crystalline cellulose although not as effective as enzymatic digestion (Gama *et al*., 1997). It has been reported that ultrasound increases the reactivity of cellulose (Aimin *et al.,* 2005), although the researchers did note that ultrasound did not impact on cellulose crystallinity.

Of the few studies that do exist, ultrasonic pre-treatment of lignocellulose has been reported as effective upon sunflower husk (Sulman *et al*., 2011). Ultrasound has also been reported as successful in the pre-treatment of rice hull, with samples treated with ultrasound showing significantly higher levels of hydrolysate sugars following enzymatic digestion than untreated samples. The researchers did however note that ultrasound was not as successful as pre-treatment with H_2O_2 (Yu *et al*., 2008). Neither Yu *et al*., (2008) or Sulman *et al*., (2011) proved the mechanism by which ultrasound was successful in pre-treating the biomass with both speculating that it was the result of the physical effects of cavitation bubble implosion upon the structure of the biomass. However the chemical effects of ultrasound must also be considered.

In addition to the physical effects of cavitation bubble implosion, ultrasound produces chemical effects through the dissociation of water to form hydroxyl and hydrogen radicals (Joseph *et al*., 2000). In addition to this ultrasound has been shown to be effective in the production of hydroxyl radicals from a variety of other oxidative chemicals such as ozone and

hydrogen peroxide (Gogate and Pandit, 2004) and this is discussed in detail in Chapter 5.

Hydroxyl radicals are thought to be one of combination of strategies that wood decay fungi employ in the degradation of lignocellulose (Eriksson et al., 1990; Wan and Li, 2012). They have been shown to degrade lignin through the oxidation of C-H bonds contained within lignin subunits (Ek, Gierer and Jansbo, 1989). Consequently it would appear logical to assume that the degradation of lignin by hydroxyl radicals could be harnessed as a potential technique for the pre-treatment of lignocellulose for bioethanol production. This centres upon the proposal that the degradation of lignin removes the barrier protecting cellulose and hemicellulose from enzymatic hydrolysis. However the use of hydroxyl radical production systems in the pre-treatment of lignocellulose is an area which has seen little attention within the primary literature.

1.10 Research aims

The application of ultrasound in both a general biotechnological context as well as its use in the field of lignocellulose derived ethanol production processes is an area that is gaining increasing attention. The following thesis details a PhD research project which sought to assess the application of ultrasound in the bioconversion of spent grains to bioethanol. The thesis is split into five distinct results chapters with each having differing aims but each contributes to the overall aim of assessing the applicability of ultrasound in the conversion process.

Specific objectives of each chapter are as follows:

Chapter 3:

- To further understand the composition of the brewery and distillery spent grains used during the study.
- To determine the maximum potentially extractable fermentable carbohydrate from spent grains, with a view to calculating conversion efficiencies of the pre-treatment methods under study.

Chapter 4:

- To evaluate the use of a widely adopted conventional lignocellulose pre-treatment method (acid hydrolysis) and assess its use in the hydrolysis of SG.
- To optimise the extraction of fermentable carbohydrate from SG in terms of pre-treatment and enzymolysis methodological paramaters such as: acid concentration, temperature, residence time and enzyme loading and duration of enzymatic digestion.
- To establish a benchmark with which to compare novel ultrasonic pre-treatment techniques

Chapter 5:

- To evaluate the use of ultrasound in the pre-treatment of spent grains
- To evaluate the use of ultrasound in combination with various chemical pre-treatments (acid, hydrogen peroxide, ozone) of spent grains

Chapter 6:

- To further understand the effects of ultrasonic irradiation upon the enzymes involved during the enzymolysis of spent grains.
- To assess the potential of ultrasound to reduce enzyme loading rates

Chapter 7:

- To assess the ability of the various yeasts, contained with the University's culture collection, in terms of their ability to ferment the sugars present within spent grain hydrolysates.

Chapter 2

Materials and methods

2. Materials and methods

2.1 Spent grain handling

Samples (50 kg) of Brewer's spent grains (BSG) were obtained from Bellhaven Brewery (Dunbar, UK) and Caledonian Brewery (Edinburgh, UK). Samples (50 kg) of distiller's spent grains (DSG) were obtained from a Scottish grain whisky distillery and a malt whisky distillery. These particular breweries and distilleries were selected in an attempt to obtain samples across the range of SG types available in Scotland. Those obtained from the breweries comprised 100% malt based SG from Caledonian brewery and 96% malt/4% roasted malt based SG from Bellhaven. SG obtained from the distilleries was 100% malt based from the malt whisky distillery and 85% maize/15% malted barley based from the grain whisky distillery. Brewing and distilling operations in Scotland usually utilise barley, maize or wheat as the source of fermentable carbohydrate. As such the SG samples utilised in the study were representative of the range of SG available across Scotland, with the exception of wheat based SG.

In order to be able to store the biomass in a reasonably stable condition, SG was dried at 50 ℃ for 48 h and then milled with a hammer mill (Retsch, Germany) fitted with a 2 mm screen. SG was stored in air tight containers at ambient temperature until required. The drying procedure produced spent grains with a moisture content of ~1% (w/w) and due to the low moisture content it was assumed that there would be limited SG degradation during storage. The research documented in following chapters used the dried and milled SG as a feed stock (with the exception of moisture analysis conducted upon whole SG as received). The stock of SG was

homogenised by manually inverting the container several times in order to gain a representative sample for each experiment.

2.2 Hydrolysate preparation

The acids used during pre-treatment of SG were HCl, H_2SO_4, HNO_3 and the base used in hydrolysate neutralisation was NaOH and these were all obtained from Fisher Scientific, UK. They were used in the preparation of solutions of desired concentration as specified later in this chapter. Following pre-treatment and subsequent enzymolysis, residual biomass was separated from the hydrolysate via vacuum filtration with glass fibre filters (Millipore, USA).

2.3 Hydrolytic enzymes

Pre-treatment and enzymolysis studies utilised a variety of hydrolytic enzymes, all of which were obtained from Novozymes (Denmark). The enzymes consisted of those contained within the Cellic digestion kit and the biomass kit. Cellic enzymes comprised both Cellic Htec and Cellic Ctec and the biomass kit enzymes comprised NS50013, NS50010, NS50030 and NS50014. Further enzyme specifics are detailed in Table 2.1. During all enzymolysis work, enzymes were dosed as specified later in this chapter and incubated in an orbital shaker (Electron incubator, Infors, UK) at 50℃ and agitation was applied at 100 rpm.

Table 2.1 Enzyme characteristics

Enzyme	Class	Activity	Action
Cellic Ctec	Cellulase & β - glucosidase complex	1000 EGU/g [a]	Hydrolyses cellulose to glucose
Cellic Htec	endo - 1, 4 - Xylanase	1000 FXU/g [b]	Hydrolyses hemicellulose to a mixture of hexoses and pentoses
NS50013	Cellulase complex	700 EGU/g [a]	Hydrolyses cellulose to cellobiose
NS50010	β - glucosidase	250 CbU/g [c]	Hydrolyses cellobiose to glucose
NS50030	Xylanase	500 FXU/g [b]	Hydrolyses xylan to xylose
NS50014	Hemicellulase	750 FXU/g [b]	Hydrolyses hemicellulose to monomeric sugars

[a] Endoglucanase units
[b] Fungal xylanase units
[c] β – glucanase units

2.4 Carbohydrate analysis

All of the sugar analysis documented in this thesis was conducted with high pressure liquid chromatography (HPLC). The HPLC setup consisted of; a Thermoseparation Products Spectra Series AS100 autosampler (Thermo Fisher Scientific, USA) installed with a Rezex ™ RHM Monosaccharide ion exchange column (Phenomenex, USA). This was coupled to a SP6040XR refractive index detector (Spectraphysics, USA). The system was linked to a

PC installed with CSW32 chromatographic station (Data Apex, Czech Republic). The software requires the building of a relevant calibration for sugars of interest, in this case cellobiose, glucose, arabinose, xylose, galactose and mannose (all from Fisher, UK). The calibration was constructed for varying concentrations of the sugars up to $10gL^{-1}$. Hydrolysates were filtered through 0.2µm syringe filters (Millipore, USA), were diluted within range of the calibration and quantified with reference to the calibration and an internal standard of known concentration (stachyose, meso-erythritol or salicin – all from Sigma Aldrich, USA).

2.5 Yeast strains and growth conditions

The yeasts employed in this research were; *Saccharomyces cerevisiae* DCLM (Kerry Ltd, Menstrie, UK), *Pichia stipitis* NCYC 1542 (National Collection of Yeast Cultures, Norwich, UK), *Klyuveromyces marxianus* NCYC 1425, *Candida shehatae* var. *lignososa* NCYC 2389 and *Pachysolen tannophilus* NCYC 614. Yeasts were maintained on YPD agar slopes consisting of; 2% glucose, 2% bacteriological peptone, 2% technical agar and 1% yeast extract (Oxoid Ltd, Hampshire, UK)

2.6 Ultrasound

2.6.1 Frequency of 20 kHz

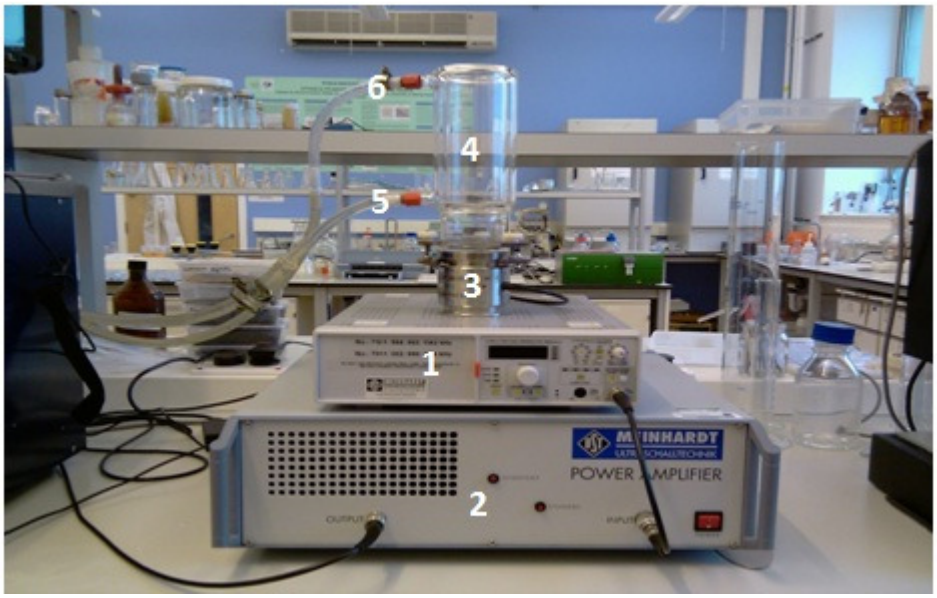
Ultrasonic pre-treatment research was conducted using a Misonix S-4000 sonication setup (Misonix, USA) (Fig. 2.1). The system operates at 20kHz and has a maximum rated power output of 600W. The setup consisted of an ultrasonic processor (containing both the signal generator and amplifier), the transducer, the ultrasonic probe and the reactor. The jacketed reaction vessel allowed cooling to be applied through the use of a thermostated circulator.

Fig. 2.1 Misonix S-400 sonicator setup, showing; 1) ultrasonic processor, 2) transducer, 3) ultrasonic probe, 4) reactor, 5) cooling water inflow, 6) cooling water outflow.

2.6.2 High frequency ultrasound

Ultrasound assisted enzymolysis research was conducted using a variable frequency mega-sonicator (Meinhardt, Germany) (Fig. 2.2). The system allows for variation in frequency between 382 – 1174kHz and has a maximum rated power output of 200W. The setup consisted of an ultrasonic signal generator, amplifier, the transducer and the reactor. The jacketed reaction vessel allowed temperature to be maintained at that which was optimal for the hydrolytic enzymes used in the study, through the use of a thermostated circulator

Fig. 2.2 Meinhardt mega-sonicator setup, showing; 1) signal generator, 2) amplifier, 3) transducer, 4) reactor, 5) cooling water inflow, 6) cooling water outflow.



2.6.3 Ozone generation

During pre-treatment studies that involved the combined use of ultrasound and ozone. A laboratory ozone generator (Ozone Engineering, USA) was used to diffuse ozone into the reactor. The generator produced ozone via corona discharge using pure oxygen as the feed gas.

2.6.4 Hydrogen peroxide

20 volumes hydrogen peroxide (Fisher, UK) was used during pre-treatment research that involved the use of H_2O_2. It was used in the preparation of solutions of desired concentration as specified later in this chapter.

2.7 Spent grain compositional analysis

2.7.1 Moisture content

The method used to analyse SG moisture content involved evaporating the moisture from a known weight of SG and measuring the weight lost. Aluminium weighing dishes (Fisher, UK) were pre-dried in a muffle furnace (Cole – Parmer, USA) at 105℃ for 2 hours. Dishes were cooled in a desiccator and weights were recorded to the nearest 0.1mg. 1g of SG was weighed into a weighing dish and the weight of the sample plus dish was recorded. Dishes were returned to the muffle furnace at 105℃ for 4 hours. Samples were removed from the furnace and cooled to room temperature in a desiccator. After cooling the weight of the dish plus sample was recorded to the nearest 0.1mg and dishes were returned to the furnace. This process was repeated until a constant weighting was achieved (constant weight being defined as less than 0.1% change in weight upon 4 hours of incubation at

105℃). Weights obtained from before and after drying were used to calculate SG total moisture content (on a wet weight basis), using the function;

$$\% \text{ Moisture} = (\text{Weight}^{a} - \text{Weight}^{b}/\text{Weight}^{a}) \times 100$$

[a] Weight of wet SG

[b] Weight of dry SG

2.7.2 Residual starch content

Starch content was analysed using a Total Starch (AA/AMG) kit from Megazymes, Ireland. The assay involved hydrolysing residual starch enzymatically and then quantifying the glucose release. The manufacturer's method uses spectrophotometry to quantify glucose, however during preliminary evaluation of starch it was found that there was a poor level of reproducibility using this method. As such glucose was subsequently quantified using HPLC which was found to improve reproducibility between replicate samples giving a greater accuracy in glucose determination.

SG (100mg) was added to a 15mL plastic centrifuge tube (Fisher Scientific, UK). This process was repeated for a starch sample of known purity and was treated identically to the unknown samples throughout the remaining analysis. Ethanol (0.2mL of 80 % v/v) was added to each sample followed by vortexing for 30s. Dimethylsulphoxide (DMSO) (2mL) was added

to each sample before being returned to the vortexer for a further 30s. The addition of DMSO causes starch gelatinisation and increases solubility thereby rendering starch amenable to enzymatic hydrolysis (McCleary, Solah and Gibson, 1994).

Tubes were then capped and placed in a boiling water bath for 5 minutes. Following boiling, 2.9mL of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer and 0.1mL of thermostable α – amylase (Megazymes, Ireland) was added to each tube. Samples were vortexed for 30s and returned to the boiling water bath for 6 minutes. During the incubation samples were vortexed for 10s at 2 minute intervals. Following incubation, 4mL of sodium acetate buffer and 0.1mL of amyloglucosidase (Megazymes, Ireland) were added. Samples were vortexed for 30s and placed in a water bath at 50℃ for 30 minutes.

Starch contains two constituent components both of which are polymers of glucose albeit with different structural arrangement of the glucose subunits. Amylose is a linear polymer comprising α1-4 linked subunits, whilst amylopectin is a branched polymer consisting of α1-4 linked glucose subunits on the linear backbone and α1-6 linked glucose branches. α-amylase is a glycoside hydrolase that cleaves α1-4 glycosidic bonds within both amylose and amylopectin, acting at random points along the starch molecule. They cannot however cleave α1-4 glycosidic bonds found between adjoining glucose subunits which contain glucose sidechains linked by α1-6 glycosidic bonds or indeed α1-6 bonds themselves (Howling, 1989). As such, in the absence of other starch hydrolysing enzymes α-limit dextrins are formed. Amyloglucosidase is another type of starch hydrolysis enzyme which

cleaves glucose subunits from the non-reducing end of the starch backbone. Additionally they also possess the ability to cleave α1-6 bonds found within amylopectin and when used in conjunction with α-amylase results in the reduction of limit dextrin formation and the conversion of starch to quantifiable glucose (McCleary, Solah and Gibson, 1994).

The Samples were then centrifuged at 4000 G for 10 min, --filtered through 0.2µm syringe filters and quantified for glucose via HPLC. Starch content (glucose equivalent) was then calculated using glucose recovery from the starch control as follows:

$$\% \text{ Recovery} = (a/b) \times 100$$

a: [glucose] from HPLC data

b: known weight before hydrolysis

The starch content for each of the SG samples was then calculated using the function:

$$\% \text{ Starch} = (a \times (b/c))/(d \times e) \times 100$$

a: glucose concentration determined by HPLC

b: total volume added to solids

c: dry weight of SG sample

d: starch oligomer correction factor (1.11) – used to adjust free D-glucose to anhydro D-glucose present in starch i.e. accounts for the addition of water to glucose as starch is hydrolysed.

e: % glucose recovery from the starch control

.

2.7.3 Total carbohydrates and lignin

SG carbohydrates and lignin were analysed using a method adapted from the National Renewable Energy Laboratory (Washington, USA), detailed in technical report NREL/TP-510-42618. The method involved hydrolysing the carbohydrate component within draff and splitting the residual biomass (containing klason lignin and ash) from the sugar containing hydrolysate. The original protocol stipulates drying the residue obtained following carbohydrate extraction for 24 h and then to continue drying until less than a 0.1% reduction in weight is observed upon 1 h of reheating. However, it was found that the residue remaining following carbohydrate extraction from SG achieved a constant weight after 4 h of drying. As such the method was adapted using 4 h drying instead of the 24 h stated in the original method. Following the drying step, the residual biomass is then combusted to ash. Total carbohydrate is quantified by analysing the sugars contained in the hydrolysate and klason lignin is determined by subtracting the weight of the ash component from the residual biomass.

2.7.3.1 Sample preparation

Gooch crucibles (Fisher Scientific, UK) were placed in a muffle furnace at 575℃ for 4h followed by cooling in a desiccator for 1h. The crucibles were weighed to the nearest 0.1 mg and the weights were recorded. A sample of SG (300mg) was added to a tared pressure tube (Fisher Scientific, UK) and the weight of sample plus the pressure tube was recorded. 72% H_2SO_4 (3mL) was added to the tube and a glass stirring rod was used to mix the sample for 1 minute. The tubes were placed in a water bath at 30℃ for 60

minutes with samples being stirred at 10 minute intervals using the glass rod. Following hydrolysis, the acid was diluted to 4% by adding dH_2O (84mL). Tubes were capped and samples were mixed by inverting the tubes for 30s. In order to assess the loss of sugars via heat degradation during the second hydrolysis step, it was necessary to quantify sugar loss by exposing a series of standard sugar solutions to the conditions of the second hydrolysis stage. This was achieved by preparing standard solutions of glucose, arabinose and xylose at concentrations of $20gL^{-1}$, $10gL^{-1}$ and $20gL^{-1}$, respectively. The concentrations of the standard solutions were intended to mimic expected concentrations of the three sugars within SG. The standard solutions were transferred to pressure tubes and along with the unknown samples were autoclaved at 121℃ for 1h. Following the second hydrolysis step, samples were allowed to cool to room temperature before being analysed for total carbohydrate and Klason lignin.

2.7.3.2 Analysis of Klason lignin and ash

Samples prepared in 2.7.3.1 were analysed for acid insoluble lignin. Hydrolysis solutions were vacuum filtered through one of the previously weighed Gooch crucibles, with the filtrate being captured in a Buchner flask and kept for HPLC analysis. A dH_2O wash bottle was used to transfer the entire hydrolysis residue into the Gooch crucible. The solids captured within the crucible were then washed with ~50mL of dH_2O. Crucibles were transferred to an incubator at 105℃ for 4h before being cooled in a desiccator for 30 minutes. After cooling the weight of the crucible and the dry residue was recorded to the nearest 0.1mg. Crucibles were transferred to a muffle furnace at 575℃ for a residence time of 24h, before being cooled in a

desiccator for 30 minutes. The weight of the Gooch crucible plus the residual ash was then recorded to the nearest 0.1mg. This was used in conjunction with previously recorded weights to calculate acid insoluble lignin, using the equation:

$$\% \text{ Klason lignin} = ((a - b) - (c - b) - d))/e \times 100$$

a: weight of gooch crucible plus dry insoluble residue
b: weight of gooch crucible
c: weight of gooch crucible plus ash
d: SG % protein content
e: weight of dry SG sample

Total SG ash was also calculated, using the function:

$$\% \text{ Ash} = (a - b)/c \times 100$$

a: weight of gooch crucible plus ash
b: weight of gooch crucible
c: weight of dry SG sample

2.7.3.3 Analysis of total carbohydrate

Hydrolysates produced in 2.7.3.1 along with the sugar recovery standards which had been exposed to the same hydrolysis conditions, were analysed for total sugar content. Calculated sugar concentrations were used to quantify total spent grain carbohydrate. Firstly, the percentage sugar

recovery of each of the sugar standards exposed to the second hydrolysis step was calculated, using the equation:

$$\% \text{ Recovery} = (a/b) \times 100$$

a: sugar concentration determined by HPLC

b: known concentration prior to 2nd hydrolysis step

The % recovery values were then used to correct the sugar concentration of the sample hydrolysates to account for sugar degradation during hydrolysis. This was done using the function:

$$\text{Corrected [sugar]} = a/(b/100)$$

a: [sugar] as determined by HPLC

b: % recovery

Corrected monomeric sugar concentrations were then used to calculate polymeric sugar concentrations through multiplication by an anhydro correction factor of 0.88 for the pentoses and 0.9 for the hexoses. Total hydrolysate polymeric sugar concentration was then calculated by addition of the individual sugar concentrations. Total SG carbohydrate was then calculated using the function:

$$\% \text{ Total Carbohydrate} = (a \times b)/c$$

a: total concentration of polymeric sugar

b: volume of liquid added during hydrolysis

c: weight of SG hydrolysed

2.7.4 Determination of spent grain protein

Protein content of SG samples was analysed utilising an alkaline protein extraction followed by total protein characterisation using a Bradford assay (Bradford, 1976). A sample of SG (2g) was weighed into a conical flask, to which 18mL of 2M NaOH was added and the sample was incubated at 65℃ for 1 hour. The residual biomass was separated from the liquid portion (containing extracted proteins) under vacuum filtration using glass microfibre filters (Millipore, USA). Protein standards of concentrations 0, 25, 125, 250, 500, 750, 1000, 1250 and 1500µg/mL were prepared using bovine serum albumin and dH_2O. A sample (30µl) of each of the SG protein samples and protein standards was transferred to a centrifuge tube and Coomassie reagent (1.5mL) was added to each sample followed by incubation at room temperature for 5 minutes. Subsequent to the incubation the absorbance of each sample was recorded at 595nm. Absorbance data from the protein standards was used in creation of a standard calibration curve which was utilised to determine protein concentrations of the spent grain samples.

2.8 Optimisation of conventional techniques for the pre-treatment and enzymolysis of spent grains

The use of acid/heat in the pre-treatment of lignocellulosic biomass has been shown to be successful in a large number of reports (Sorensen *et al.*, 2008; Deprez *et al.*, 2009; Yoswathana *et al.*, 2010). Process parameters, such as acid concentration, temperature and duration of heat treatment have been reported as important in determining the efficiency of pre-treatment and

subsequent enzymolysis. With this in mind an experimental methodology for the optimised pre-treatment of BSG and DSG was developed, by investigating the effects of these parameters upon the efficiency of acidic pre-treatment.

2.8.1 Variables affecting the efficiency of dilute acid pre-treatment

2.8.1.1 Type of acid

Three differing acid species (H_2SO4, HNO_3 and HCl) were assessed in terms of their ability to pretreat spent grains. SG was mixed with acid at a concentration of 0.2N and a total solids loading of 10% w/w. Samples were pre-treated via a thermal cycle with parameters of 120 ℃ and 30 PSI for 20 mins. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using 10M NaOH and hydrolysates were sampled for sugar analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively and samples were incubated at 50℃ for 24h. Subsequent to enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment. Experimental controls consisted of SG samples mixed with water (final pH ~6.5) and exposed to the thermal treatment followed by enzymatic digestion (pH adjusted to 5-5.3 following heat treatment) and untreated SG digested enzymatically (mixed with water, pH adjusted to 5-5.3 before enzyme dosing).

2.8.1.2 Acid concentration

In order to further optimise the use of acid in the pre-treatment of SG, the effects of varying acid molarity upon pre-treatment efficiency was assessed. SG was mixed with HNO_3 at a concentration of 0.2, 0.5, 1 or 2M and a total solids loading of 10% w/w. Samples were pre-treated via a thermal cycle with parameters of 120 ℃, 30 PSI for 20 minutes. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using 10M NaOH and hydrolysates were sampled for later analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Subsequent to enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment. Experimental controls consisted of SG samples mixed with water and exposed to the thermal treatment and untreated SG digested enzymatically.

2.8.1.3 Temperature and duration of thermal decomposition

The next stage in the optimisation of the use of acid to pre-treat SG, involved assessing the effects of the temperature and duration of the thermal cycle applied during pre-treatment. SG was mixed with HNO_3 at 1 M with a total solids loading of 10% w/w. Samples were pre-treated via a thermal cycle with parameters of 100, 120, or 140℃ at 30PSI with residence times of 20, 40 and 60 min for each of the temperatures. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using 10M NaOH and hydrolysates were sampled for sugar analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g

biomass), respectively. Samples were incubated at 50℃ for 24h. Subsequent to enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment.

Whilst feasible at the lab scale, a 24 h enzyme incubation time could prove to be uneconomic upon scale up of ligocellulosic ethanol production to industrial levels. Enzyme costs and lengthy incubation time are one of the major challenges that need to be circumvented before main stream production of 2nd generation ethanol becomes a reality. Enzyme manufacturers continue to bring new cellulolytic enzymes to market and shortened incubation times are one of the advantages specified when compared to previous enzyme preparations (e.g. Novozymes new Cellic release – Ctec 3 and Htec 3).

2.8.2 Variables effecting carbohydrate release during enzymolysis

2.8.2.1 Enzyme dosing and duration of enzymolysis

Two of the keys parameters that are assessed when evaluating the efficiency of cellulolytic enzymes in the release of sugars from lignocellulose are dosing levels and duration of enzymatic digestion. Desired enzyme characteristics are low dosing levels and a rapid digestion. Consequently, Cellic Ctec and Htec were assessed for hydrolytic efficiency by optimising dosing levels and residence time in terms of enzymatic digestion of pretreated SG.

SG was mixed with HNO_3 (0.2M) at a total solid loading of 10% w/w and samples were pre-treated via a thermal cycle with parameters of 120℃ and 30PSI for 20 minutes. Following pre-treatment, sample pH was adjusted

within the range 5-5.3 with 10M NaOH and hydrolysates were analysed for sugar content. Cellic enzymes were added with Ctec and Htec dosed at 1% and 0.167%, 6% and 1% or 12% and 2% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50 ℃ for 72h, with samples being taken every 24h for sugar analysis. The experimental control consisted of SG exposed to the acid hydrolysis and then subsequently incubated at 50℃ for 72h without the addition of enzyme.

2.8.3 Optimised methodology for the extraction of carbohydrate using conventional techniques

The results obtained from conventional pre-treatment and enzymolysis research provided an optimised process for the extraction of fermentable sugars from both BSG and DSG. This was used to calculate the maximum possible sugar extraction using acid and heat and provided a benchmark with which to compare novel pre-treatment methods discussed later in the thesis. Percentage conversion efficiency was calculated using the total carbohydrate data obtained during SG compositional analysis using the function:

% Conversion = (actual sugar extraction/ total carbohydrate content) x 100

2.9 Effect of ultrasound on spent grains

2.9.1 Ultrasonic pre-treatment of spent grains

The use of ultrasound as lignocellulose pre-treatment methodology is an emerging technology with little reported in the primary literature as to the factors influencing its success. However, it is likely that two critical parameters are ultrasonic output power and residence time and these were

evaluated in terms of their effects upon efficiency in the pre-treatment of BSG and DSG

2.9.1.1 Influence of ultrasonic power

SG was mixed with dH2O at a total solids loading of 10% w/w within the reactor and exposed to ultrasound with a frequency of 20 kHz, a duty cycle of 100%, a residence time of 1h and an output power of approximately 30, 60 or 120 W. The temperature was maintained at 50℃ by circulating water through the reaction vessel jacket using a thermostated circulator. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M) and hydrolysates were sampled for sugar analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Following enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment. The experimental control consisted of SG mixed with dH_2O, incubated in the reactor at 50℃ without exposure to ultrasound and exposed to the same enzymatic digestion parameters as the ultrasound treated samples.

2.9.1.2 Influence of residence time

SG was mixed with dH2O at a total solids loading of 10% w/w within the reactor and exposed to ultrasound with a frequency of 20kHz, a duty cycle of 100%, an ultrasonic output power of 120W and a residence time of 1, 3 or 5 h. The temperature was maintained at 50℃ by circulating water through the reaction vessel jacket using a thermostated circulator. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using 10M NaOH

and hydrolysates were sampled for sugar analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Following enzymolysis, hydrolysates were sampled and analysed for carbohydrate content along with samples taken following pre-treatment. The experimental control consisted of SG mixed with dH2O, incubated in the reactor at 50℃ without exposure to ultrasound and exposed to the same enzymatic digestion parameters as the ultrasound treated samples.

2.9.2 Combined ultrasonic and chemical pre-treatment of SG

2.9.2.1 Dilute acid pre-treatment in combination with ultrasound

SG was mixed with HNO_3 (0.2M) at a total solids loading of 10% w/w. As discussed in Chapter 4 optimisation of acid treatment research found that 1M nitric acid was the most efficient of the acid concentrations evaluated in pre-treating SG. As such a concentration of 0.2M was selected in order to evaluate the use of ultrasound in improving the effectiveness of acid pre-treatment in relation to reducing the required concentration of acid.

The reaction mixture was added to the reactor and the sample was exposed to ultrasound with a frequency of 20kHz, a duty cycle of 100%, an ultrasonic output power of 120 W and a residence time of 1, 3 or 5h. The temperature was maintained at 50℃ by circulating water through the reaction vessel jacket using a thermostated circulator. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M) and hydrolysates were sampled for sugar analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec

at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Following enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment. The experimental control consisted of SG mixed with HNO_3 (0.2M), incubated in the reactor at 50℃ without exposure to ultrasound and exposed to the same enzymatic digestion parameters as the ultrasound treated samples.

2.9.2.2 Hydrogen peroxide pre-treatment in combination with ultrasound

Hydrogen peroxide at a concentration of 1% has been shown to be effective in the pre-treatment of lignocellulose (Gould, 1985). Consequently, SG was mixed with 1% H_2O_2 at a total solids loading of 10% w/w within the reactor and exposed to ultrasound with a frequency of 20kHz, a duty cycle of 100%, an ultrasonic output power of 120 W and a residence time of 1, 3 or 5h. The temperature was maintained at 50℃ by circulating water through the reaction vessel jacket using a thermostated circulator. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M) and hydrolysates were sampled for later analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Following enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment. The experimental control consisted of SG mixed with 1% H_2O_2, incubated in the reactor at 50℃ without exposure to ultrasound and exposed to the same enzymatic digestion parameters as the ultrasound treated samples.

2.9.2.3 Ultrasound assisted ozonolysis of spent grains

SG was mixed with dH_2O at a total solids loading of 10% w/w. The reaction mixture was added to the reactor and exposed to a combined ultrasound and ozone pre-treatment. This involved bubbling ozone through the reactor at a flow rate of 5L min^{-1} and exposing the sample to ultrasound with a frequency of 20kHz, a duty cycle of 100%, an ultrasonic output power of 120 W and a residence time of 1, 3 or 5h. The temperature was maintained at 50℃ by circulating water through the reaction vessel jacket using a thermostated circulator. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M) and hydrolysates were sampled for later analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Following enzymolysis, hydrolysates were sampled and analysed for sugar content along with samples taken following pre-treatment. The experimental control consisted of SG mixed with dH_2o, incubated in the reactor at 50℃ and exposed to ozone without exposure to ultrasound. Samples were then treated to the same enzymatic digestion parameters as the ultrasound treated samples.

2.9.2.4 Ultrasound assisted ozonolysis of spent grains in combination with hydrogen peroxide

SG was mixed with 1% H_2O_2 at a total solids loading of 10% w/w. The reaction mixture was added to the reactor and exposed to a combined ultrasound and ozone pre-treatment. This involved bubbling ozone through the reactor at a flow rate of 5L min^{-1} and exposing the sample to ultrasound

with a frequency of 20kHz, a duty cycle of 100%, an ultrasonic output power of 120W and a residence time of 1, 3 or 5h. The temperature was maintained at 50℃ by circulating water through the reaction vessel jacket using a thermostated circulator. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M) and hydrolysates were sampled for later analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Samples were incubated at 50℃ for 24h. Following enzymolysis, hydrolysates were sampled and analysed for carbohydrate content along with samples taken following pre-treatment. The experimental control consisted of SG mixed with 1% H_2O_2, incubated in the reactor at 50℃ and exposed to ozone without exposure to ultrasound. Samples were the treated to the same enzymatic digestion parameters as the ultrasound treated samples

2.10. Effects of ultrasound on the enzymes involved in lignocellulose hydrolysis

Initial investigation into the effects of ultrasound upon cellulolytic enzyme function was conducted using a simplified substrate (cellulose powder). As discussed in Chapter 1 enzymatic digestion of lignocellulose usually relies on at least three types of enzyme – cellulase, β-glucosidase and xylanase (Bommarius *et al*., 2008). This made it difficult to assess the effects of ultrasound upon each enzyme. Consequently, cellulose powder was chosen for preliminary studies in order to limit the number of enzymes within the reactor. This helped to give a better understanding of the effects of ultrasound upon each enzyme.

Experimentation sought to evaluate the effect of ultrasound upon combined cellulase/ β-glucosidase digestion of cellulose to glucose. Without the presence of β-glucosidase cellulase hydrolyses cellulose to cellobiose and a lower quantity of glucose (due to cellobiose not being hydrolysed to glucose by β-glucosidase). Consequently, the next stage of research sought to further simplify the enzymatic digestion and assessed the effect of ultrasound upon the enzymolysis of cellulose by only cellulase and quantifying both glucose and cellobiose. The final stage of research using a simplified substrate involved evaluating the effects of ultrasound upon β-glucosidase hydrolysis of an aqueous cellobiose solution. The experimental methodology was finally applied to pre-treated SG using cellulase, β-glucosidase and xylanase.

2.10.1 Effects of ultrasound upon cellulase/β-glucosidase hydrolysis of cellulose

2.10.1.1 Influence of ultrasonic frequency

Micro-crystalline cellulose Avicell PH-101 (Sigma – Aldrich, USA) was mixed with dH_2O at a loading of 10% total solids (w/w), added to the ultrasonic reactor and dosed with 0.89mL cellulase (Celluclast NS50013) and 5mL β – glucosidase (NS50010). This equated to an enzyme loading of 1875 EGU/l and 37500 CBU/l for cellulase and β–glucosidase, respectively. β–glucosidase dosing was high to avoid the well documented cellobiose inhibition of cellulose (Bommarius *et al.*, 2008) . Enzymatic digestions were exposed to ultrasound at a power of 3W and a variety of ultrasonic frequencies (584, 862, 998, 1174kHz) in continuous sonication mode.

Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through the use of a thermostated circulator. Enzymatic digestions were allowed to proceed for 5h with samples taken hourly for sugar analysis.

Two experimental controls were applied. The first consisted of an identical experimental procedure except that no enzymes were dosed. The second consisted of an identical experimental procedure apart from the fact that the digestion was not exposed to ultrasound.

2.10.1.2 Influence of ultrasonic power

Micro-crystalline cellulose Avicell PH-101 (Sigma – Aldrich, USA) was mixed with dH_2O at a loading of 10% total solids (w/w), added to the ultrasonic reactor and dosed with 0.89mL cellulase (Celluclast NS50013) and 5mL β – glucosidase (NS50010). This equated to an enzyme loading of 1875 EGU/l and 37500 CBU/l for cellulase and β–glucosidase, respectively. Again, β–glucosidase dosing was high to avoid the well documented cellobiose inhibition of cellulase. Enzymatic digestions were exposed to ultrasound with a frequency of 998kHz at a power of 1.5, 3 or 6W. Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through the use of a thermostated circulator with samples withdrawn hourly for sugar analysis. Two experimental controls were applied. The first control consisted of an identical experimental procedure except that no enzymes were dosed. The second control consisted of an identical experimental procedure apart from the fact that the digestion was not exposed to ultrasound.

2.10.2 Effects of ultrasound upon cellulase hydrolysis of cellulose

2.10.2.1 Influence of ultrasonic frequency

Micro-crystalline cellulose Avicell PH-101 (Sigma – Aldrich, USA) was mixed with dH_2O at a loading of 10% total solids (w/w), added to the ultrasonic reactor and dosed with 0.89mL cellulase (Celluclast NS50013). This equated to a cellulase loading of 1875 EGU/l. Enzymatic digestions were exposed to ultrasound at a power of 3W and a variety of ultrasonic frequencies (584, 862, 998, 1174kHz) in continuous sonication mode. Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through the use of a thermostated circulator. Enzymatic digestions were allowed to proceed for 5h with samples taken hourly for sugar analysis. Two experimental controls were applied. The first consisted of an identical experimental procedure except that no enzyme was dosed. The second consisted of an identical experimental procedure apart from the fact that the digestion was not exposed to ultrasound.

2.10.2.2 Influence of ultrasonic power

Micro-crystalline cellulose Avicell PH-101 was mixed with dH_2O at a loading of 10% total solids (w/w), added to the ultrasonic reactor and dosed with 0.89 mL cellulase (Celluclast NS50013). This equated to an enzyme loading of 1875 EGU/l. Enzymatic digestions were exposed to ultrasound with a frequency of 998 kHz at a power of 1.5, 3 or 6W. Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through the use of a thermostated circulator with samples withdrawn hourly for sugar analysis. Two experimental controls were applied. The first control consisted of an identical experimental

procedure except that no enzymes were dosed. The second control consisted of an identical experimental procedure apart from the fact that the digestion was not exposed to ultrasound.

2.10.3 Effects of ultrasound upon β-glucosidase hydrolysis of cellobiose

2.10.3.1 Influence of ultrasonic frequency

Cellobiose solution (20g L^{-1}) was added to the ultrasonic reactor and dosed with 1mL β–glucosidase (NS50010). This equated to an enzyme loading of 7500 CBU/l. Enzymatic digestions were exposed to ultrasound at a power of 3 W and a variety of ultrasonic frequencies (584, 862, 998, 1174kHz) in continuous sonication mode. Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through the use of a thermostated circulator. Enzymatic digestions were allowed to proceed for 5h with sampling hourly for sugar analysis.

Two experimental controls were applied. The first was designed to assess the effect ultrasound has upon the cellobiose itself and prove that any effect displayed by the ultrasound was as a result of action upon the enzymes and not the substrate itself. This control consisted of an identical experimental procedure except that no enzymes were dosed, with the cellobiose solution exposed to ultrasound. The second control consisted of an identical experimental procedure apart from the fact that the digestion was not exposed to ultrasound.

2.10.3.2 Influence of ultrasonic power

Cellobiose solution (20 g l^{-1}) was added to the ultrasonic reactor and dosed with 1mL β – glucosidase (NS50010). Enzymatic digestions were exposed were to ultrasound with a frequency of 862 kHz at a power of 1.5, 3 or 6W. Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through the use of a thermostated circulator and were allowed to proceed for 5h with sampling occurring hourly for sugar analysis. Two experimental controls were applied. The first control consisted of an identical experimental procedure except that no enzymes were dosed. The second control existed simply to provide a benchmark with which to compare enzymatic digestions exposed to ultrasound. This control consisted of an identical experimental procedure apart from the fact that the digestion was not exposed to ultrasound.

2.10.3.3 Effects of ultrasound upon Cellic hydrolysis of spent grains

SG was mixed with H_2SO_4 (0.2M) and a total solids loading of 10% w/w. Samples were pre-treated via a thermal cycle with parameters of 120℃ and 30PSI for 20 min. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M) and hydrolysates were sampled for sugar analysis. Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively. Enzymatic digestions were exposed to ultrasound at a power of 3W and a variety of ultrasonic frequencies (584, 862, 998, 1174kHz) in continuous sonication mode for 24h. Enzymatic digestions were carried out in a jacketed ultrasonic reaction vessel with the temperature maintained at 50℃ through

the use of a thermostated circulator and sampled periodically for sugar analysis.

Two experimental controls were used to validate the data from enzymatic digestions exposed to ultrasonic irradiation. The first consisting of enzymatic digestions in the absence of ultrasound and the second consisting of exposure of pre-treated SG to ultrasound in the absence of enzyme. Other than this, controls were exposed to an identical experimental method as the ultrasonically irradiated digestions documented previously.

2.11 Fermentation of spent grain hydrolysates

As discussed in chapter one the most widely used yeast in an industrial setting (*S. cerevisiae*), does not possess the ability to ferment the pentose sugars within SG hydrolysates. Fermentation work sought to assess the pentose utilising yeasts identified within the University of Abertay yeast culture collection, alongside *S. cerevisiae*, in terms of their ability to produce ethanol from SG hydrolysate sugars.

In order to obtain a high yield of ethanol during fermentation lignocellulose hydrolysates should contain as high a concentration of sugar as possible (White *et al.,* 2008). As detailed in Chapter 5, pre-treatment using ultrasound was not as effective as pre-treatment using heat and acid. Further optimisation of the use ultrasound in the pre-treatment of lignocellulose is required to assess whether or not in can be as effective as acid treatment. This could involve the utilisation of high power equipment in excess of that used in the study. As the highest sugar concentrations were achieved through the use of conventional pre-treatment methods (combined

acid/ heat treatment) hydrolysates were prepared using conventional methods (White *et al*., 2008) in order to achieve higher ethanol yields.

2.11.1 Hydrolysate preparation

SG was mixed with HNO_3 (1 M) and a total solids loading of 10% w/w. Samples were pre-treated via a thermal cycle with parameters of 120℃ and 30PSI for 20 minutes. Following pre-treatment, sample pH was adjusted within the range 5-5.3 using NaOH (10M). Enzymatic digestion was initiated by dosing the hydrolytic enzymes Cellic Ctec and Htec at 6% and 1% w/w (g enzyme/g biomass), respectively, incubating at 50℃ for 24h with samples being removed periodically and analysed for sugar content.

2.11.2 Preparation of yeast inoculum

Yeast slopes were used to inoculate 150 mL of liquid YPD medium (2% glucose, 2% bacteriological peptone, 1% yeast extract) in Erlenmeyer flasks. Cultures were grown at 30℃ on a rotary shaker at 150 r.p.m. Cultures were allowed to grow for 48h at which point cells were washed twice in sterile distilled water, viability being determined, and used for fermentation of SG hydrolysates.

2.11.3 Fermentation

Hydrolysate samples were separated into 80 mL aliquots in sterile 100 mL Schott bottles. Yeasts were inoculated with an initial cellular density of 10 x 10^6 cells mL^{-1}. Fermentations were conducted at 30℃ and 100 rpm. and sampled periodically for 5 days. Samples were centrifuged at 13,000G and the supernatant was separated from the yeast pellet in order to cease

fermentation and stabilise the sample for further analysis. Samples were quantified for sugar and ethanol content. Yeast pellets were re-suspended in sterile water and analysed for viability and cell growth.

2.11.4 Monitoring of fermentation efficiency

2.11.4.1 Sugar utilisation

Samples taken during the course of the fermentation were analysed for sugar content using the HPLC setup detailed earlier in this chapter. Sugar concentrations from the various samples were used alongside the initial sugar concentration of the hydrolysate to calculate yeast sugar utilisation.

2.11.4.2 Ethanol content

Fermentation samples were analysed for ethanol concentration using a Shimadzu QP2010 gas chromatograph mass spectrometer (GC-MS) installed with an Agilent HP blood alcohol capillary column (ID: 0.32mm, length 7.5m, film 25µm). All samples were analysed using 1-propanol as an internal standard at a final concentration of 1%.

2.11.4.3 Yeast growth and viability

Fermentation samples were analysed for yeast cell growth and viability using a haemocytometer and staining with citrate methylene violet (0.01% methylene violet plus 2% sodium citrate).

2.12 Statistical considerations

All of the experimentation conducted during this research was done in triplicate. As such all of the data presented are the means of the analysis conducted upon the triplicates and their associated standard deviations. Tests for statistical significance were also conducted where appropriate.

Chapter 3

Spent grain compositional analysis

3. Spent grain compositional analysis

3.1 Introduction

The brewing and distilling industries generate large amounts of various types of co-products. These being mainly spent grains, pot ale, spent lees, spent wash and spent hops. SG, in terms of weight, are the most abundant co-products produced by both the brewing and distilling industries, with various studies putting their generation at between 31–39% of the original cereal weight used during the mashing process (Townsley, 1979). Both brewer's and distiller's spent grains are produced in abundance across the globe. In terms of BSG the EU alone produces ~3.4 million tons annually of which the UK contributes 0.5 million tons (Stojceska et al., 2008). This compares with Brazil, the world's fourth largest beer producer, which generates ~1.7 million tons annually (Mussatto et al., 2006). In addition to BSG, sources of DSG are also plentiful with USA alone producing ~33.3 million tons annually (Hoffman and Baker, 2012).

The SG utilised in this study were from two Scottish ale breweries, a Scottish grain distillery and a Scottish malt distillery. The breweries and the malt distillery produce a 100% malted barley based SG, whilst the grain distillery produces a maize based SG (85% maize & 15% malted barley). Maize based spent grains are particularly relevant as a feed stock for 2nd generation bioethanol production as much of the 1st generation bioethanol produced in the USA is derived from maize. Little exists in the primary literature with regard to the composition of maize based distillery SG. However a number of papers exist profiling the composition of malted barley based brewery SG (Santos *et al*., 2003; Robertson *et al*., 2010; Mussatto *et*

al., 2004; Russ *et al*., 2005; Niemi *et al*., 2012; Carvalheiro *et al*., 2004) and malted barley based distillery SG (Ranhotra *et al*., 1982)

3.1.1 Production of spent grains from malted barley based feedstocks

Barley is one of world's most widely cultivated crops, coming behind only wheat, maize and rice, in terms of gross tonnage produced annually (Kendal, 1994). The grain is characterised as having high levels of starch and protein with dry mass figures of ~74% and ~11% respectively (Jones, Cadenhead and Livingstone, 1968). The grain consists of three major components (Fig. 3.1), these being the germ (or embryo), the endosperm (consisting of both the aleurone and starchy endosperm) and the external shell which protects the interior components. The shell can be further subdivided into three components; the seed coat, the pericarp and the husk. The husk provides a layer of protection to the grain and consists mainly of lignocellulose and protein (Lewis and Young, 1995).

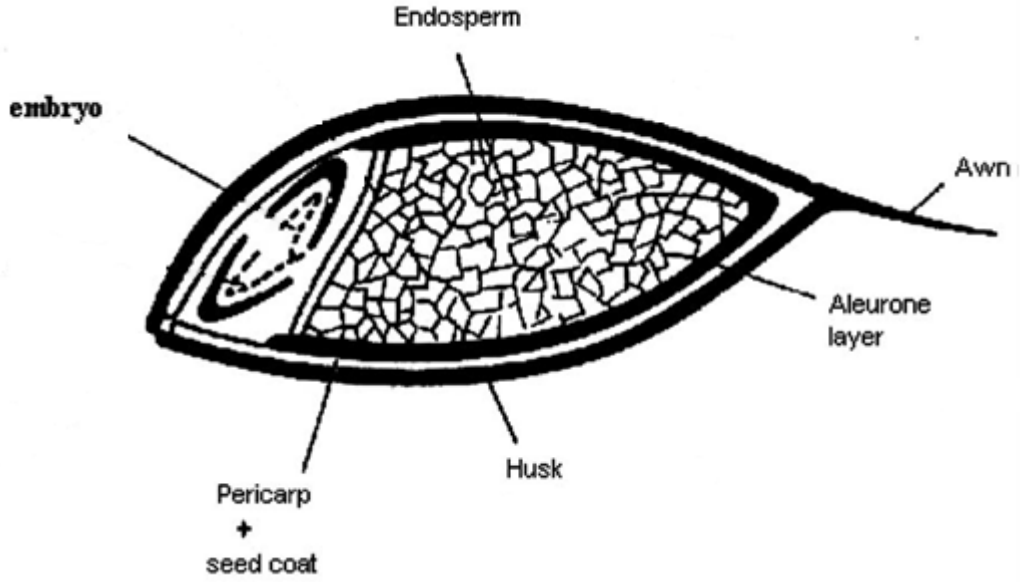


Fig. 3.1 Structure of the barley grain (adapted from Mussato *et al*., 2006)

3.1.1.1 Malting

In breweries and distilleries that use malted barley as a feedstock, the barley grain undergoes a controlled germination process, which causes the grain to express the amylase enzymes that are responsible for breaking down starch to fermentable sugars in a process called malting. Typically, the grains are steeped in tanks containing water at around 18℃ for two days. This results in the grain water content rising from ~8% to ~ 50% and initiates germination. Subsequent to steeping the barley in transferred to the germination tank where the temperature is maintained at ~ 20℃ and the humidity is kept high. The germination process typically lasts 5 days and causes the expression of various genes which promote the synthesis and activation of various enzymes in the aleurone and starchy endosperm, including; amylases, proteases and β – glucanases. During malting, β-glucanses act to degrade cell wall glucans within the barley, in a process similar to cellulase degradation of cellulose during enzymolysis of spent grains. Additionally, there is also a limited action of amylase and protease degradation of starch and protein, respectively. Following germination the grains (now referred to a green malt) are kilned at between 50 – 60℃ until they reach ~ 4% moisture, resulting in production of the malt itself.

3.1.1.2 Spent grain production in a brewery

In a brewery the malted barley is milled to form grist, which is then added to water in a vessel called the mash tun. The water is initially mixed in at a temperature of ~ 37℃, which is then raised to around 65℃. The overall process is called mashing and is utilised to promote the enzymatic digestion of the various components within malt, through the enzymes that were

produced during malting. Starch is broken down to fermentable sugars, mainly glucose, maltose and maltotriose and longer polymeric carbohydrates that cannot be fermented (dextrins) (Mussato *et al*., 2004). During mashing proteins are degraded to polypeptides and amino acids. The mashing process ultimately produces a liquid, known as wort, which is then utilised as the fermentation medium for the production of beer. Following wort production the residual solid component of the malted barley grain, primarily consisting of the husk, pericarp and seed coat components of the original cereal grains (Mussato *et al*., 2004), are allowed to settle in the mash tun. This forms a bed which the wort is allowed to filter through in a process known as lautering. Subsequent to wort filtration the residual solid cereal component is now referred to as brewer's spent grains (BSG).

3.1.1.3 Spent grain production in a malt whisky distillery

As is usually the case with a brewery, malt whisky distilleries use 100% barley malt as the source of fermentable carbohydrate in the production of whisky. There are however a number of processing differences between a brewery and a malt whisky distillery, which mainly centre upon the methods of sugar extraction from malt.

The process begins with the milling of the malt to form grist. The milling specifications vary dependent upon the distillery and the efficiency of the mash tun. Typically the roller mill is set so as to produce grist comprising 20% husk, 70% grits and 10% flour for a traditional mash tun with a finer grind for lauter mash tuns (Dolan, 2003). Following milling, the grist is fed to a steels masher and mixed with water (~4 parts water to 1 part grist) which is heated to maintain a final temperature of ~65℃ (Barnes and Andrews,

1998). The mix is then fed into the mash tun and thoroughly mixed for ~20 minutes before being allowed to stand for ~1 h. During this time amylase and protease enzymes within the malted barley convert starch and protein to fermentable monosaccharides and amino acids, respectively. Subsequently the first worts are drained through the mash bed (comprising the grist) until it is almost dry. In contrast to a brewery, a malt distillery continues to add water after the 1st worts have been allowed to run off. A second batch of water is mashed in, the temperature is raised to ~70℃ and the wort is again allowed to run off. The procedure is repeated for a third time with the temperature being raised to ~80℃ (Dolan, 2003). The wort collected during the mashing process is used as the fermentation medium in the production of malt whisky. Subsequent to wort filtration the residual solid cereal component is now referred to as distiller's spent grains (DSG). As is the case with SG produced from malted barley within a brewery, it mainly comprises the husk, pericarp and seed coat components of the original cereal grains (Mussato *et al*., 2004).

3.1.2 Production of spent grains from maize based feedstocks

Maize is one of the world's most widely cultivated crops with global annual production of ~800 million tons (Lobell, Schlenker and Costa-Roberts, 2011). Much of current production is located with North America with the United States accounting for ~140 million tons of global production annually. A large proportion of US maize production (~40%) is currently diverted to the production of 1st generation bioethanol (Dien *et al*., 2002). As such, research which seeks to utilise maize based SG as a feedstock for bioethanol

production is of significant importance as it has the potential to increase ethanol yields within the American fuel alcohol industry.

The maize kernel has four principal components (Fig. 3.2), those being the endosperm (containing the starch), the germ, the pericarp or hull and the tip cap. The maize grain differs from barley in a number of aspects. It contains similar amounts of starch comprising ~72% of the dry weight of the grain. However it contains significantly less fibre (2.2%) and protein (8.8%) (Watson, 1984). The lower fibre content would be expected to correlate with lower lignin content both within the grain itself as well as spent grain derived from it.

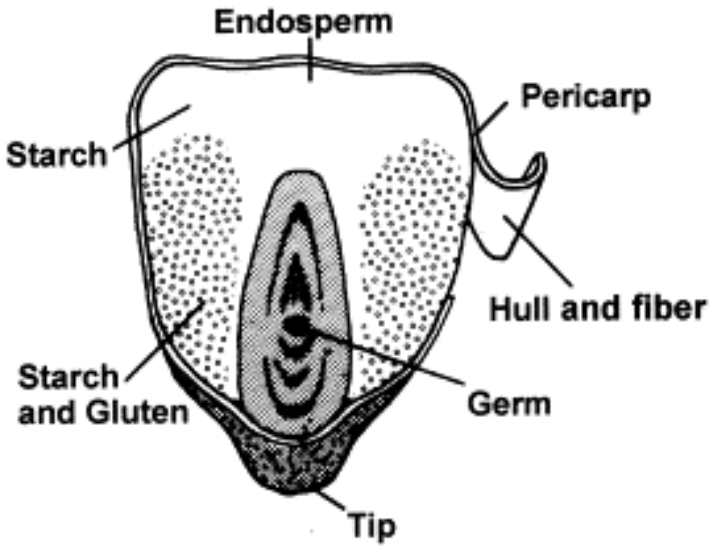


Fig. 3.2 Structure of the maize grain (adapted from Shukla and Cheryan, 2001)

3.1.2.1 Production of spent grains in a grain whisky distillery

The processes involved in the production of spent grains within a grain whisky distillery differ significantly from either a brewery or a malt whisky

distillery. Typically, grain whisky distilleries use malted barley in combination with another cereal as the source of fermentable carbohydrate, usually maize (Bathgate and Cook, 1989) or wheat (Brown, 1990). Other cereals such as barley, triticale and rye are used less frequently (Lyons and Rose, 1977). The spent grains used in this study were derived from a grain distillery mash that comprised 85% maize and 15% malted barley.

As is the case in both a brewery and a malt whisky distillery the production of a fermentable wash begins with milling of malted barley and unmalted cereal. This is done in order to increase water penetration during the cooking stage (Kelsall and Lyons, 1999). Starch within unmalted cereals is ultimately hydrolysed by the amylase enzymes that are present within the malted barley component of the mash. Due to the fact that a grain distillery mash comprises a majority of grains that are unmalted they require additional processing before mashing. This typically involves high temperature cooking which gelatinizes the starch and renders in amenable to amylase digestion.

The milled unmalted cereal is mixed with water in the slurry tank at a ratio of ~2.5 litres per tonne of cereal (Piggott and Conner, 1995), with the temperature being maintained at around 40℃ above ambient. The slurry is then pumped to the cooker which is typically a pressure vessel which possesses stirring equipment. Steam is injected and the temperature is raised to 130-150℃ (Pyke, 1965) and held for ~20 minutes. The cooking process results in the dissociation and uncoiling of the helical regions of amylose, reduction of amylopectin crystallinity and hydration and swelling of the starch granule (French, 1984). This renders the starch amenable to

digestion by the amylase enzymes presents within the malt component of the mash.

Following cooking the cooked slurry is discharged into the conversion tank/ mash tun and malted barley slurry that has been held at around 40℃ is added. The temperature is maintained at 62-65℃ for 30 minutes (Robson, 2001). The principle function of conversion is to allow the starch that has been gelatinized during cooking, alongside the starch within the malt, to be converted to fermentable sugars (glucose, maltose and maltotriose). Additionally, proteins are degraded to amino acids which provide essential nutrients to the fermenting yeast. As is the case with starch hydrolysis, protein degradation is achieved by the endogenous proteolytic enzymes contained within the malt component of the mash.

Modern grain distilleries typically now operate a “grains in” process whereby the spent grains are pumped to the fermenter along with the wort. However the grain distillery from which spent grains were obtained for this study is currently operating using the traditional method. This involves draining the wort through the mash tun and sparging the grains several times with water at increasing temperature. Following wort separation the residual grain component is now referred to as distiller’s spent grains.

3.1.3 Effect of processing differences upon composition and structure of SG

The differences in cereal processing between a brewery, malt whisky distillery and grain whisky distillery would be expected to have an effect on both the composition and structure of the spent grains they produce.

Additionally the composition of spent grains will vary dependent upon the cereal grain from which they are derived.

Brewery SG would be expected to contain significantly more starch than those from either a malt or grain whisky distillery. As outlined previously, the mashing process conducted within either type of distillery utilises a number or repeated hot water extractions to maximise extraction of fermentable carbohydrate. This is in contrast with a brewery which adds a single batch of mashing water followed by a single sparging with hot water. The differences in processing lead to more effective starch hydrolysis and sugar recovery within a distilling process than a brewery process. As such compositional characterisation of SG from a brewery and distillery would be expected to confirm a higher residual starch component within BSG when compared to DSG.

The processing differences between breweries and distilleries might also be expected to affect the physical structure of the lignocellulose component within SG. In particular the cooking process conducted within a grain distillery process ultimately exposes the draff to a prior pre-treatment stage to which SG from a brewery is not exposed. Coupled with this malt distilleries utilise a number of hot water sparges with temperature in excess of those seen in a typical brewery mashing process. The use of liquid hot water at temperatures between 130-180°C has been shown to partially degrade lignin and hemicellulose in a number of instances (Bobleter, 1991; Kohlmann *et al*., 1995; Laser *et al*., 2002; Yang and Wyman, 2004). The temperature range that grain distilleries employ when cooking cereal (130-150°C) falls directly within the range at which liquid hot water has been

shown to be effective in partial lignin and hemicellulose degradation. As such grain distillery processing is likely to lead to the partial breakdown of the lignocellulose structural matrix within spent grains, thereby improving the effectiveness of methods used to further extract fermentable carbohydrate (i.e. pre-treatment and enzymolysis). This effect is likely to be less pronounced in SG obtained from a malt distillery, as whilst the grains have been subjected to temperatures in excess of that utilised within a brewery, they fall some way short of those used during grain cooking within a grain distillery.

3.1.4 Current and potential applications of brewer's and distiller's spent grains

SG are usually sold as cattle feed, although other applications are gaining increasing attention within the brewing and distilling industries, including; use as a source of lignocellulosic biomass for the production of bioethanol (White *et al*., 2008), use as a combustion feedstock for the production of steam or electricity (Patel *et al*., 1996), a feedstock for the production of bio-coal (bio-char) and bio-oil via pyrolysis (Sanna *et al*., 2011; Wang *et al*., 2012) or use as a source of biodegradable carbon in the creation of biogas via anaerobic digestion (Ezeonu and Okaka, 1996; Bochmann *et al*., 2007)

3.2 Experimental approach

Whilst a significant amount of literature exists, that profiles the composition of both BSG and DSG, there appears to be a level of variation in composition, dependent upon the source of SG and the cereal grain from which they were derived. In order to further understand the particular characteristics of the SG

used in this study, analysis was conducted with a view to assessing various compositional parameters, including total water, starch, extractable carbohydrate, protein, klason lignin and ash.

Limited visual assessment was conducted. However it was noted prior to milling that maize based spent grains from the grain distillery had a smaller particle size than either of the brewery SG or malt distillery SG. It was also lighter in colour indicating heat exposure during the cereal cooking process conducted within a grain distillery. SG obtained from the breweries and malt distillery had a similar particle size and colour. This would be as expected due to the fact that both the brewery and malt distillery SG was derived from 100% barley malt and neither had been exposed to the high temperatures to which the grain distillery SG had. Further visual assessment could include analysis by electron microscopy which would give further detail of the structural differences between the types of SG under study.

3.3 Results and Discussion

In terms of SG total water content (Fig. 3.3) data showed that there was a level of similarity between SG, when considering those from distilleries and breweries, regardless of the source of spent grains. This equated to total water contents of 82.15, 78.65, 81.23 and 79.15% (w/w) for SG samples from Bellhaven Brewery, the grain distillery, Caledonian Brewery and the malt distillery, respectively. These results are similar to moisture contents reported by other studies. Robertson *et al.*, 2010 reported the moisture content of BSG to be 75-80 % (w/w), Santos *et al.*, 2003 found the moisture contents of BSG that was sampled from ten different distilleries to be 76.8 –

78.9% (w/w), whilst other studies have found the moisture content to be between 77 – 81% (w/w) (Mussatto *et al.*, 2004; Russ *et al.*, 2005).

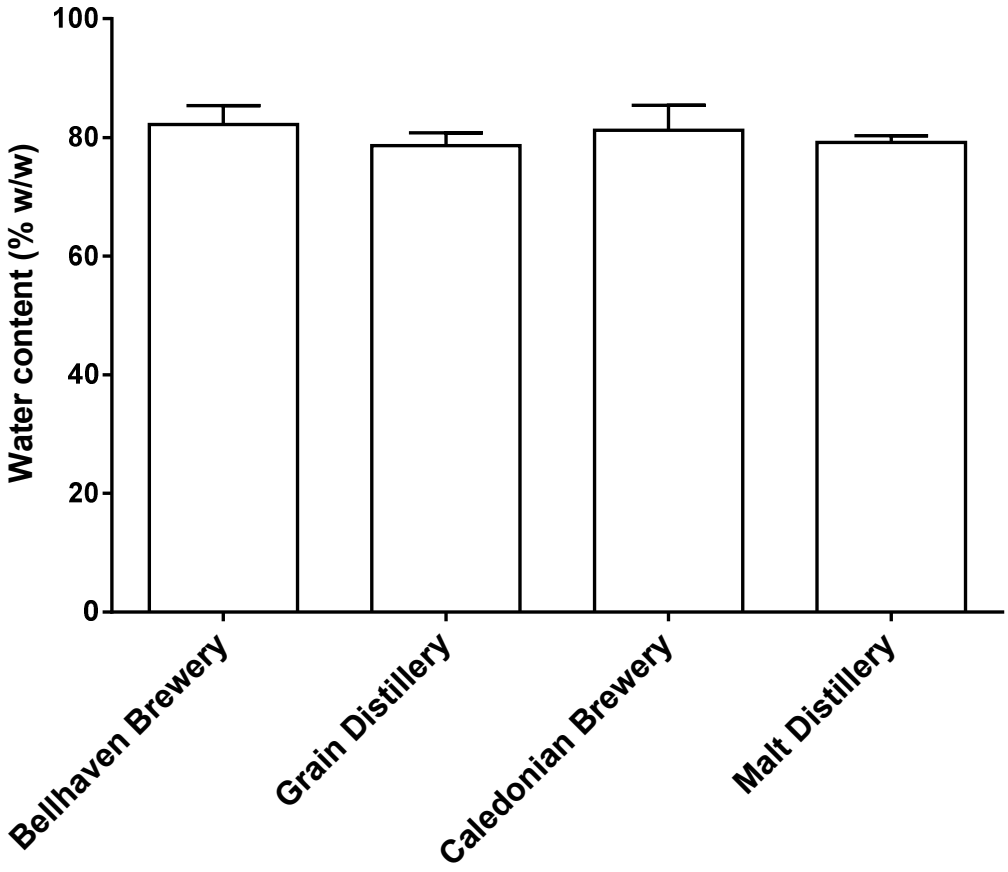


Fig. 3.3 Spent grain moisture content

Starch analysis (Fig. 3.4) showed that there was a high degree of variability between distillery and brewery SG. Starch levels comprised 3.87, 2.01, 4.18 and 1.95 (% w/w dry SG) for SG samples from Bellhaven Brewery, the grain distillery, Caledonian Brewery and the malt distillery, respectively. In general, BSG appears to have higher residual starch content than DSG, this can be attributed to differences in processing between breweries and distilleries. Malt distilleries and grain distilleries typically

conduct three or more hot water extractions at increasing temperature during the mashing procedure. This is in contrast to a brewery which adds mashing water only once (or twice if sparging is employed). The process conducted within distilleries leads to more efficient starch extraction and hydrolysis and as such brewery SG would be expected to contain more starch.

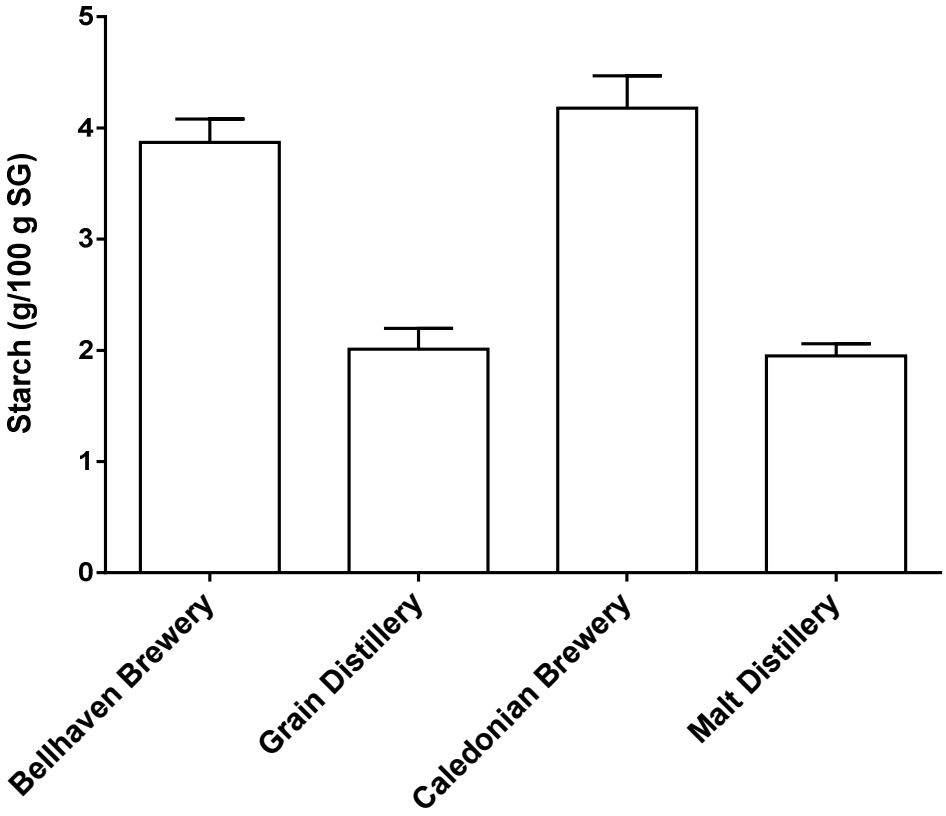


Fig. 3.4 Spent grain residual starch

The carbohydrate content of lignocellulosic biomass is one of the key parameters in defining whether or not it is suitable as a substrate for bioethanol production, with high levels of extractable carbohydrate being desirable. Analysis for total potentially extractable carbohydrate (Fig. 3.5)

also showed a degree of variability between SG samples; 51.36, 56.75, 46.63 and 42.15% (w/w dry SG) for SG samples from Bellhaven Brewery, the grain distillery, Caledonian Brewery and the malt distillery, respectively. These results appear similar to previously reported values for the maximum carbohydrate content of SG; 47.2% (w/w dry SG) (Kanauchi *et al*., 2001), 45.2% (w/w dry SG) (Mussato *et al*., 2005), 44.4% (w/w dry SG) (Jay *et al*., 2008), 38.4% (Robertson *et al*., 2010), 46.7% (Niemi *et al*., 2012), 40% (Santos *et al*., 2003) and 51.5 % (Carvalheiro *et al*., 2004). Whilst SG total carbohydrate appears to fall in broadly similar range, there appears to be slight variation dependent of the source of the SG as well as the cereal grain from which it has been derived.

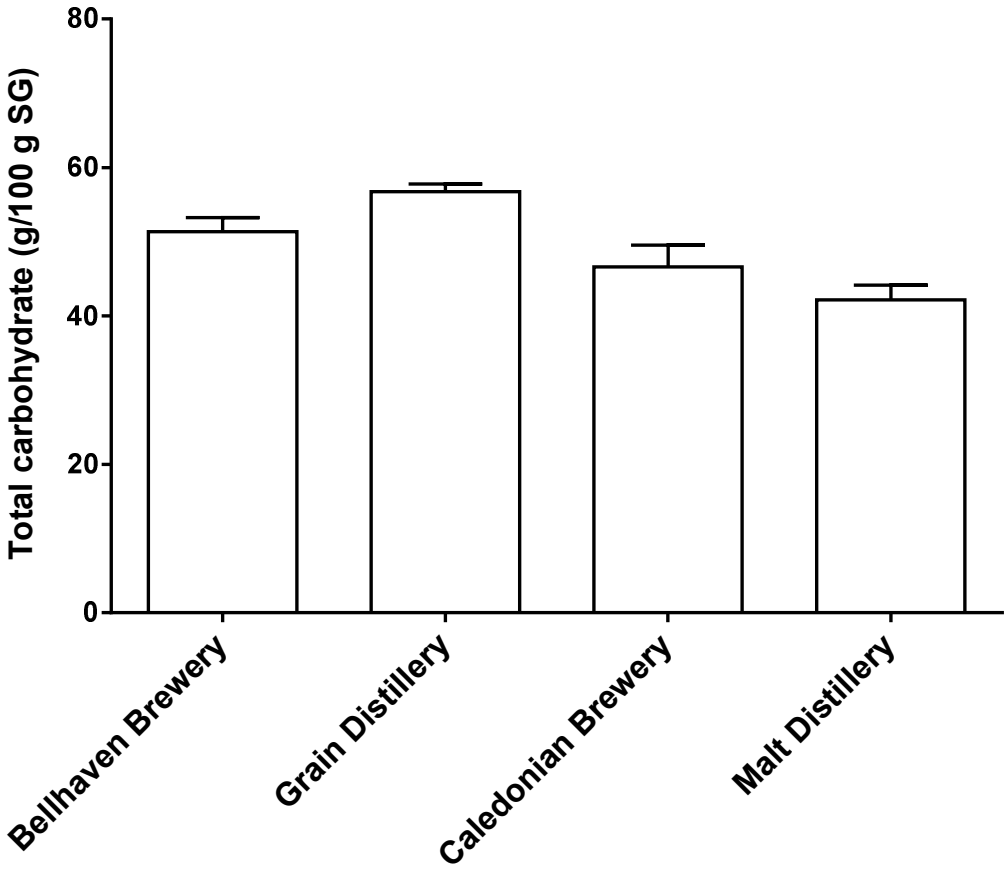


Fig. 3.5 Spent grain total extractable carbohydrate

Lignin is the primary component that complicates the extraction of sugars from lignocellulose, as such low lignin levels are highly desirable in a feedstock for bioethanol production. Lignin analysis (Fig. 3.6) conducted on SG samples showed levels of Klason lignin to be 22.19, 25.16, 19.71 and 23.15% (w/w dry SG) for SG samples from Bellhaven Brewery, the grain distillery, Caledonian Brewery and the malt distillery, respectively. Again, the results appear broadly similar to previously reported values for SG klason lignin content - 11.9% (w/w dry SG) (Kanauchi *et al*., 2001), 27.8% (w/w dry SG) (Mussato *et al*., 2005), 14.4% (Robertson *et al*., 2010), 19.4% (Niemi *et al*., 2012), 16% (Santos *et al*., 2003) and 21.7% (Carvalheiro *et al*., 2004). Of the four types of SG characterised, the maize based SG displayed the highest lignin content, with the barley based SG tending to have lower lignin contents. This is unexpected as maize typically has lower levels of fibre than barley (Watson, 1984) which in turn confers lower lignin content. However the unexpected results could likely be attributed to the similar lignin content in all of the SG under study and the large standard deviations observed with replicate analysis.

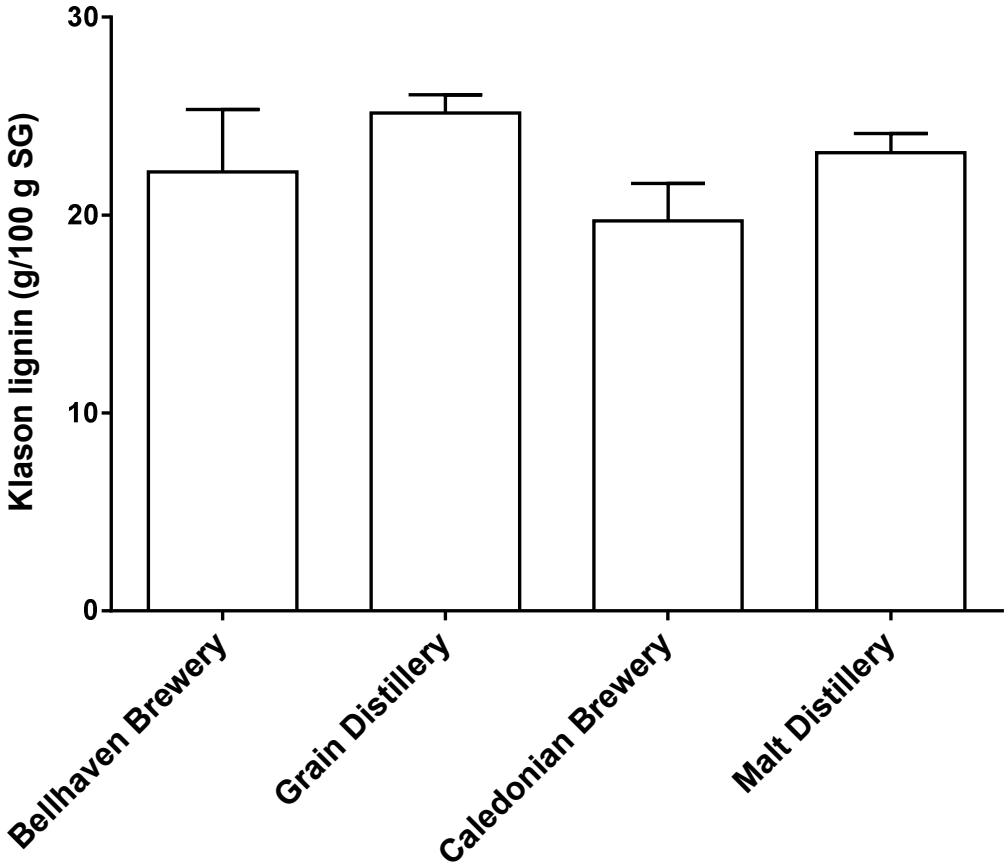


Fig. 3.6 Spent grain Klason lignin content

Protein content is an important characteristic within a lignocellulosic feedstock that is to be used in bioethanol production. This is mainly due to the fact that hydrolysed proteins are the only source of amino acids to the yeast employed in fermenting hydrolysate sugars. Protein analysis (Fig. 3.7) showed total protein content of SG to be 22.5, 15.8, 19.71 and 23.86% (w/w dry SG) for SG samples from Bellhaven Brewery, the grain distillery, Caledonian Brewery and the malt distillery, respectively. These values are broadly similar to the range of protein contents reported by other researchers; 15.2% (Mussato *et al*. 2005), 18.62% (Robertson et al., 2010)

and 23.3% (Niemi *et al*., 2012). Protein levels tended to be higher in the malted barley based SG than was observed in the maize based SG.

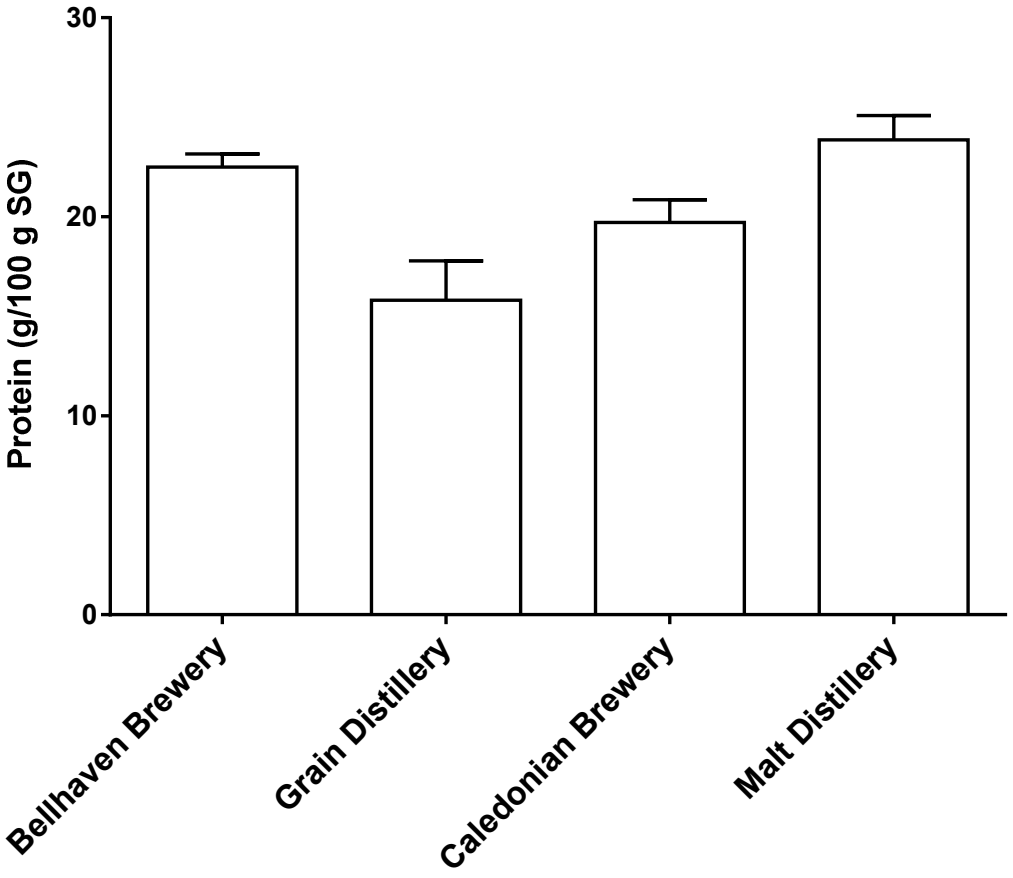


Fig. 3.7 Spent grain protein content

Ash content analysis (Fig. 3.8) showed total ash to be 1.17, 1.81, 0.94 and 1.65% (w/w dry SG) for SG samples from Bellhaven Brewery, the grain distillery, Caledonian Brewery and the malt distillery, respectively. These values are similar to SG ash content reported by Carvalheiro *et al*., 2004 who reported value of 1.2%. However, they are lower than other reported values of; 2.4% (Kanauchi *et al*., 2001), 4.6% (Mussato *et al*., 2005), 4.9% (Niemi *et al*., 2012) and 4% (Santos *et al*., 2003).

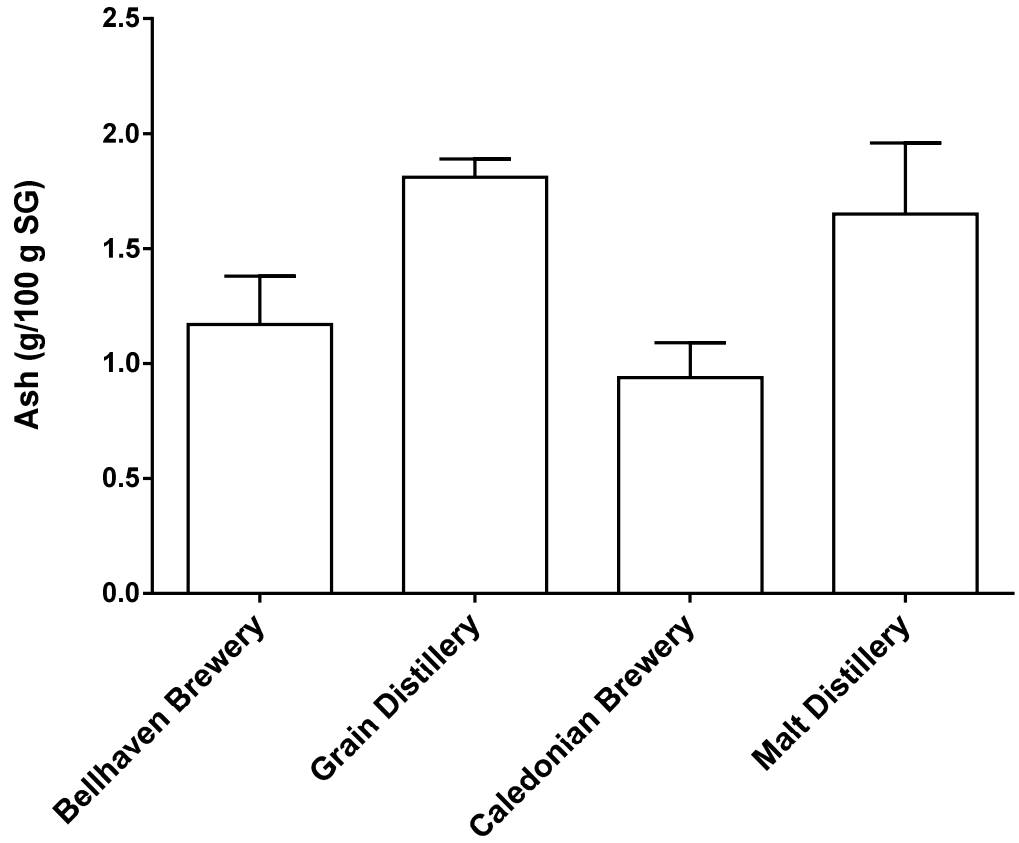


Fig. 3.8 Spent grain ash content

Whilst there appears to be a degree of variation, both between SG used in this study and previously reported values, this can attributed to expected variations between both the source of SG and also variation between cereal plant species. In general the composition of both BSG and DSG reported here is broadly similar to previously reported values. As should be expected there is a differing composition between maize and barley based SG. Maize based DSG from North British distillery had more extractable carbohydrate, less protein and higher lignin content that barley malt based BSG obtained from both of the breweries and the malt distillery. It was interesting to note that malted barley based BSG had significantly more extractable carbohydrate than malted barley based DSG.

3.4 Summary

- The SG samples assessed all displayed large amounts of extractable carbohydrates and protein.
- Whilst there is a degree of variation both between the SG used in this study and other reports, the majority of SG compositional parameters fall within a broadly similar range.
- There is a degree of variability in SG composition dependent on source of SG and also the cereal grain from which they are derived.
- SG are considered suitable feedstock for bioethanol production.

Chapter 4

Pre-treatment and enzymolysis of spent grains

4. Pre-treatment and enzymolysis of spent grains

4.1 Introduction

One of the main aims of the research conducted during this project was to evaluate the applicability of ultrasound in the pre-treatment of lignocellulose, using spent grains as a bioethanol feedstock. Many studies exist looking at the optimisation of conventional pre-treatment techniques (e.g. hydrothermal, acidic or alkaline treatment) on a large variety of lignocellulosic substrates, including; rice straw (Yoswathana *et al*, 2010), microalgae (Harun *et al*, 2011), Miscanthus grass (Sorensen *et al*, 2008), oil seed rape straw (Mathew *et al*, 2011), Eucalyptus wood (Romani *et al*, 2010), wheat straw, hay and poplar (Deprez *et al*, 2009). The use of acid in the pre-treatment of lignocellulose is one of the most widely utilised approaches, having been a focus of research for decades (Ruttan, 1909; Faith and Hall, 1944; Harris et al., 1945). A wide variety of acids are effective in degrading lignin, hydrolysing hemicellulose and increasing the susceptibility of cellulose to enzymatic digestion (Mosier *et al*., 2005)

At the start of this project spent grains were a relatively untested substrate for bioethanol production. It had been proven they are a significant source of carbohydrate (Carvalheiro et al., 2004; White *et al*, 2008; Mussato and Roberto, 2005), however there was relatively little in the primary literature with regards to optimised processes for the extraction of fermentable carbohydrate from spent grains. In addition to this, there was a need to have a bench-mark with which to compare the efficiency of ultrasonic pre-treatment.

White *et al*., (2008) exposed BSG to acid pre-treatment and compared a variety of acids (Sulphuric, Hydrochloric and Nitric) and concentrations in terms of their ability to pre-treat BSG prior to subsequent enzymolysis. The researchers reported that pre-treatment with acid at a concentration of 0.08N was effective in releasing sugars from BSG and that of the three acids assessed HNO3 was the most effective. The researchers also noted that doubling the concentration of Nitric acid to 0.16N increased the effectiveness of the pre-treatment and subsequent sugar release during enzymolysis.

Carvalheiro et al., (2004) exposed to BSG to pre-treatment with dilute (3%) sulphuric acid at 130℃ and evaluated the effects of increase residence time upon the effectiveness of sugar extraction. The researchers concluded that increasing pre-treatment residence time increase the release of sugars from BSG. However they also noted that a tipping point was reached whereby increasing residence time further caused a decrease in hydrolysate sugars and that this decrease was attributable to sugar loss through heat degradation. Mussato and Roberto, (2005) exposed BSG to sulphuric acid pre-treatment and investigated the effects of varying acid concentration upon the release of sugars. The researchers concluded that sulphuric acid was effective in the pre-treatment of BSG and the release of sugars. They also noted that increasing sulphuric acid concentration resulted in an increase in the levels of extracted glucose but a decrease in the pentose sugars (arabinose and xylose).

4.2 Experimental approach

The approach of this chapter was to investigate the following aspects of spent grains conversion to fermentable sugars: lignocellulose structural changes during pre-treatment, acid concentration, hydrolysis residence times and enzyme loading and duration of enzymolysis. These are all deemed important in determining the rate and efficiency of the hydrolysis methodology (Zhu *et al*., 2006). Research was therefore conducted looking to optimise the use of a combined heat and dilute acid pre-treatment of spent grains followed by enzymatic digestion to extract carbohydrates. This was achieved by assessing the abilities of a variety of acids, at varying concentrations, for their ability to pre-treat spent grains. This approach was further optimised by conducting studies into the effects of temperature and residence time upon the efficiency of combined acidic and thermal pre-treatment. Further studies sought to optimise enzymolysis by comparing various enzyme preparations as well as dosing levels and enzymatic digestion residence time.

Of the four sources of spent grains assessed for compositional characterisation detailed in Chapter 3, two were selected for use in the optimisation of sugar extraction utilising acid pre-treatment in combination with enzymatic digestion. The samples selected for study were spent grains from the grain distillery (referred to as DSG in subsequent data) which were derived from 85% maize/15% malted barley and those from Bellhaven Brewery (referred to as BSG in subsequent data) which were derived from 100% barley malt. The SG from the grain distillery was selected due to the fact they contain a large maize component, a feedstock which has particular

relevance for the American bioethanol industry which currently has the largest capacity for bioethanol production globally. As such research which seeks to increase ethanol production from maize based feedstocks are of current topical importance. Samples of SG from the ale brewery were selected for logistical purposes in that the research group had large quantities available.

4.3 Results and discussion

4.3.1 Variables affecting the efficiency of dilute acid pre-treatment

4.3.1.1 Type of acid

All of the acids assessed in terms of their ability to pre-treat SG, were found to be efficient in hydrolysing SG hemicellulose fractions to monomeric sugars, during the thermal stage of the pre-treatment process. This was characterised by the prevalence of the pentose sugars arabinose and xylose within the hydrolysate. All of the acids also possessed the ability to degrade the lignin fraction and increase accessibility to the cellulose fibre, leaving them susceptible to further enzymatic hydrolysis. However, the level of pre-treatment efficiency was found to differ between acid species. This was true for both brewer's spent grains (BSG) and distiller's spent grains (DSG).

Regarding DSG, total sugar concentration within the hydrolysate following pre-treatment (Fig. 4.1), varied depending on the type of acid with which it was pre-treated. HNO_3 was the most effective in hydrolysing sugars from DSG and showed a greater release of glucose, arabinose and xylose (Table 4.1). All of the acids under study showed a greater sugar release than

the control (pre-treated with water and heat), suggesting acid pre-treatment of DSG is more effective than treatment with hot water.

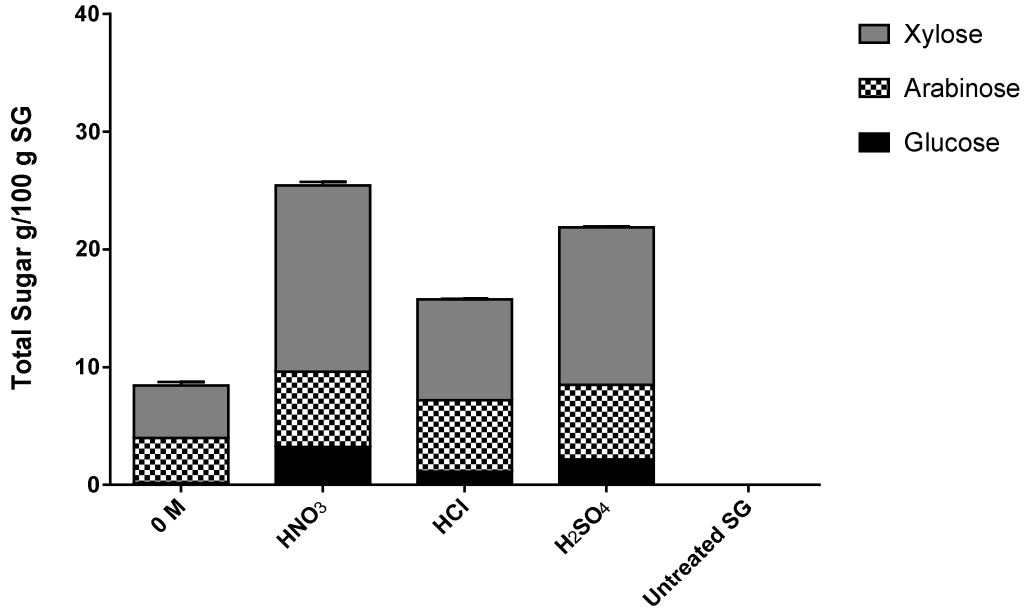


Fig. 4.1 Effects of acid type upon sugar release during 0.2M acidic pre-treatment of DSG (Results are means of triplicate treatments with SD for total sugar)

Table 4.1 Effects of acid type upon specific sugar release during pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis)

Acid type	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	**Arabinose**	**Xylose**	**Total**
HNO_3	3.21 (0.113)	6.39 (0.059)	15.84 (0.18)	25.44 (0.294)
HCL	1.12 (0.009)	6.08 (0.014)	8.55 (0.058)	15.75 (0.054)
H_2SO_4	2.18 (0.027)	6.32 (0.026)	13.38 (0.052)	21.88 (0.090)
Control	0.18 (0.064)	3.8 (0.092	4.48 (0.197)	8.46 (0.291)
Untreated DSG	0 (0)	0 (0)	0 (0)	0 (0)

The effects of pre-treatment upon the structure of lignocellulose are three-fold. These being: the hydrolysis of the hemicellulose fraction to a mixture of pentose and hexose sugars, the degradation of lignin thereby increasing cellulose accessibility and the decrease of cellulose crystallinity. The latter is required as the crystalline structure of cellulose makes enzyme accessibility difficult in areas other than those which are amorphous in nature. When considering the pre-treatment efficiency of the three acids used in this study it is clear that all three possessed the ability to hydrolyse the hemicellulose fraction within DSG. However, HNO_3 appeared significantly more efficient than either HCl or H_2SO_4**,** yielding the greatest concentration of pentose sugars within the hydrolysate.

The increase in pentose sugars was more apparent for xylose with little difference between acids in terms of arabinose release. The hemicellulose fraction of SG mainly comprises arabinoxylan (White *et al*., 2008) which consists of a back-bone of xylose sub-units with arabinose branches. Arabinose has been shown to have a higher thermal sensitivity than xylose and for this reason is released first from the hemiccellulose fibre (Carvalheiro *et* al., 2004). As such, it would appear that the combination of acid and heat used in this study resulted in the removal of arabinose branches with differences between acids mainly being due to differences in their ability to hydrolyse the xylose back-bone. It is likely that HNO_3 was more effective in hemicellulose hydrolysis due to the fact that, of the three acids assessed, HNO_3 is the most highly oxidising.

Glucose levels within the hydrolysates following pre-treatment can be attributed to three factors. Firstly, as expected, the hemicellulose fraction

within spent grains that is hydrolysed during pre-treatment contains low levels of glucose. Secondly, the acidic pre-treatment, having hydrolysed lignin and hemicellulose, also hydrolysed small of amounts of cellulose. Thirdly, a portion of glucose levels following pre-treatment are the result of the hydrolysis of residual starch left behind during the mashing process.

The experimental methodology employed did not assess lignin degradation or changes to cellulose crystallinity directly. These factors were assessed indirectly by observing levels of hydrolysate sugars present subsequent to enzymatic hydrolysis. Both lignin degradation and cellulose crystallinity have an effect upon cellulolysis, in that poor lignin degradation or failure of the pre-treatment to decrease cellulose crystallinity inhibit enzymatic hydrolysis of cellulose. It follows that an assessment of the efficiency of enzymatic digestion is an indirect method to assess pre-treatment efficiency in terms of lignin degradation and decreasing cellulose crystallinity.

However the experimental methodology could have been expanded to quantify lignin degradation and changes to cellulose crystallinity directly. Quantification of lignin degradation products such as vanillin, vanillic acid, coniferyl and sinapyl alcohol would give a more accurate determination of the levels of observed lignin degradation and a greater understanding of the efficiency of each of the acids in degrading lignin. Quantification of lignin degradation products could have been achieved with either HPLC or GC-MS (Pecina *et al*., 1986). Quantification of cellulose crystallinity could have been conducted by nuclear magnetic resonance (NMR) spectroscopy (Liitia *et al*., 2003) and would give a greater understanding of the effects of each acid

type upon cellulose crystallinity and their potential to increase the enzymatic digestibility of the cellulose fraction within SG.

Total sugar levels rose following enzymatic digestion and the level of sugar release varied depending upon the acid with which it has been pre-treated (Fig. 4.2). DSG pre-treated with HNO_3 showed the greatest release of total sugar release during enzymolysis and displayed increased yields of glucose, arabinose and xylose (Table 4.2). Lignin degradation is required before efficient enzymatic digestion can occur and the data suggests that HNO_3 was the most effective in degrading lignin. It was interesting to note that glucose was the majority sugar hydrolysed during enzymolysis and that enzymatic liberation of the pentose sugars was limited. This suggests that the majority of hemicellulose hydrolysis occurs during the pre-treatment stage.

Table 4.2 Effects of the acid used in pre-treatment upon specific sugar release during enzymolysis of DSG (Results are means of triplicate treatments with SD in parenthesis)

Acid type	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	**Arabinose**	**Xylose**	**Total**
HNO_3	16.89 (0.639)	7.46 (0.266)	18.39 (0.638)	42.74 (1.52)
HCL	16.43 (1.241)	6.75 (0.337)	13.77 (0.769)	36.95 (2.33)
H_2SO_4	16.82 (0.162)	6.9 (0.112)	16.05 (0.341)	39.77 (0.774)
Control	8.16 (0.131)	4.53 (0.068)	5.21 (0.057)	17.9 (0.245)
Untreated DSG	1.15 (0.086)	0.25 (0.044)	0.19 (0.039)	1.59 (0.135)

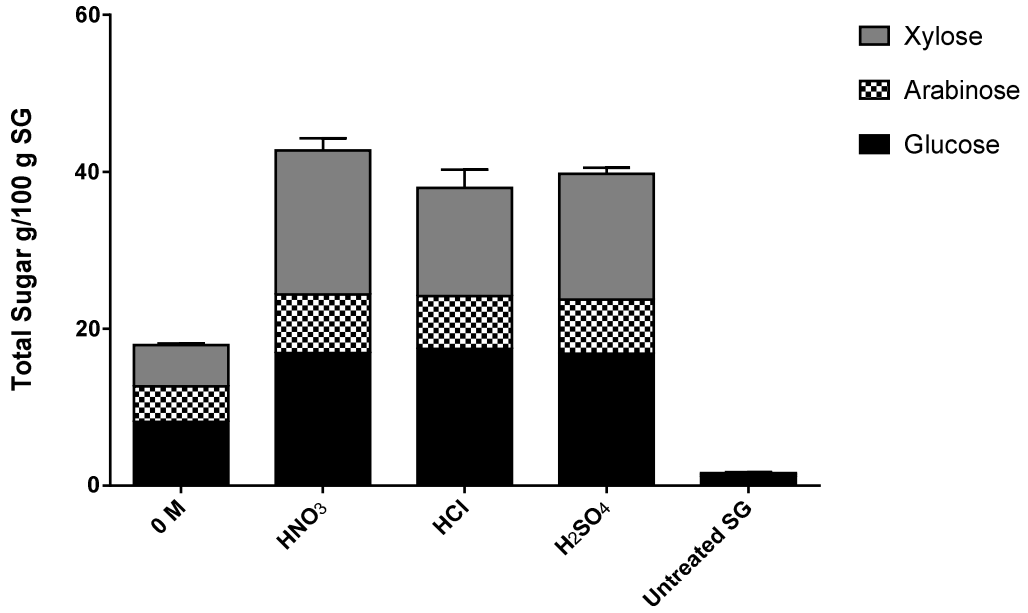


Fig. 4.2 Effects of the acid type upon sugar release during enzymolysis of 0.2M acid pre-treated DSG (Results are means of triplicate treatments with SD for total sugar)

Regarding BSG, total sugar concentration within the hydrolysate following pre-treatment (Fig. 4.3), was variable depending upon the acid utilised during pre-treatment, for samples treated with HNO_3, HCl and H_2SO_4. As was observed with DSG, HNO_3 was the most effective in release of sugar during pre-treatment of BSG and showed greater levels glucose, arabinose and xylose release (Table 4.3).

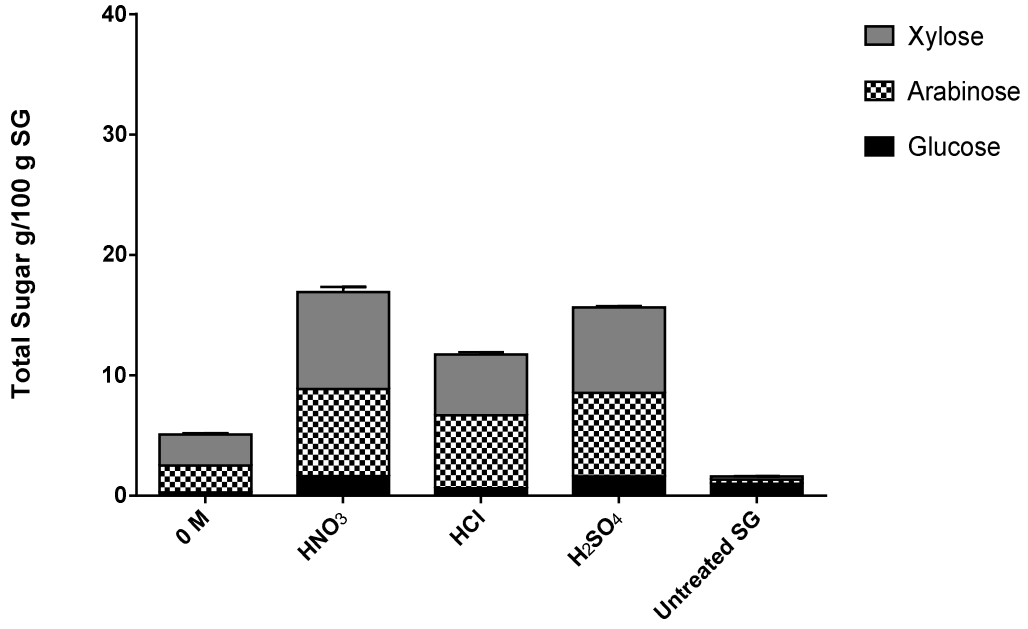


Fig. 4.3 Effects of acid type upon sugar release during 0.2M acidic pre-treatment of BSG (Results are means of triplicate treatments with SD for total sugar)

Table 4.3 Effects of acid type upon specific sugar release during pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis)

Acid type	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
HNO_3	1.64 (0.091)	7.26 (0.059)	8.06 (0.286)	16.96 (0.425)
HCL	0.62 (0.012)	6.05 (0.289)	5.07 (0.122	11.74 (0.173)
H_2SO_4	1.59 (0.029)	6.97 (0.06)	7.1 (0.064)	15.66 (0.101)
Control	0.27 (0.011)	2.22 (0.061)	2.59 (0.053)	5.08 (0.117)
Untreated DSG	0.16 (0.002)	0.73 (0.005)	0.65 (0.004)	1.54 (0.007)

During enzymolysis of BSG, sugar levels rose dependent upon which acid they had been pre-treated with (Fig. 4.4). As previously observed with DSG, HNO_3 appeared to be the most effective in rendering BSG amenable to enzymatic sugar extraction. This translated to an increased yield of glucose, arabinose and xylose (Table 4.4) compared to enzymolysis of BSG pre-treated with H_2SO_4 or HCl and significantly more than sugars released during enzymatic digestion of the control sample exposed to combined water and heat treatment. There was a limited release of sugars from untreated DSG as expected.

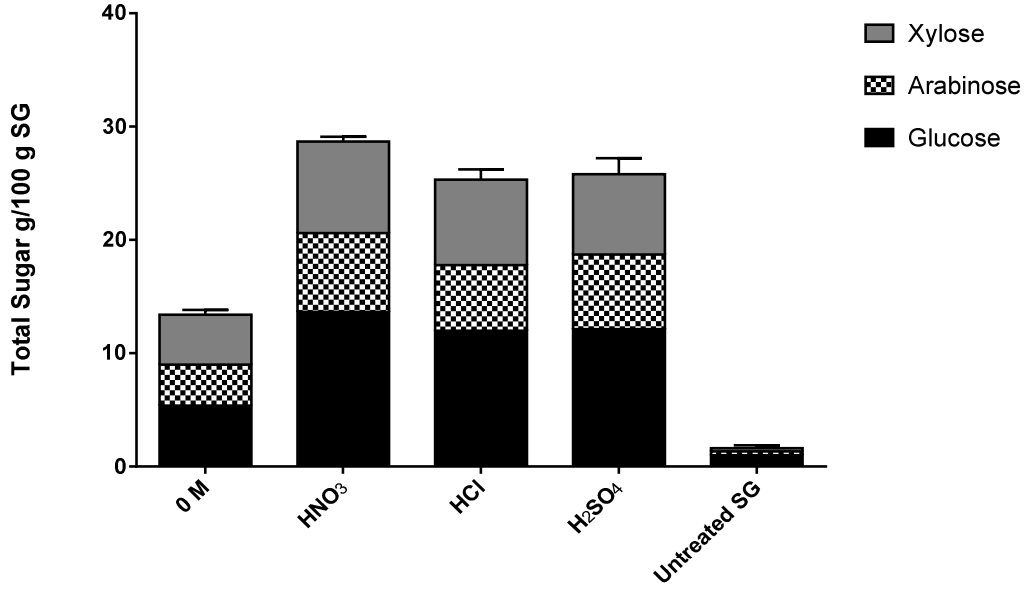


Fig. 4.4 Effects of acid type upon sugar release during enzymolysis of 0.2M acid pre-treated BSG (Results are means of triplicate treatments with SD for total sugar)

Table 4.4 Effects of the acid used in pre-treatment upon specific sugar release during enzymolysis of BSG (Results are means of triplicate treatments with SD in parenthesis)

Acid type	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
HNO_3	13.63 (0.612)	6.97 (0.314)	8.06 (0.496)	28.66 (0.425)
HCL	11.99 (0.364)	5.8 (0.289)	7.54 (0.27)	25.33 (0.889)
H_2SO_4	12.12 (1.182)	6.58 (0.542)	7.1 (0.779)	25.8 (1.387)
Control	5.38 (0.101)	3.62 (0.174)	4.4 (0.237)	13.4 (0.410)
Untreated DSG	0.99 (0.086)	0.33 (0.063)	0.29 (0.072)	1.61 (0.235)

In the case of enzymolysis of both BSG and DSG, samples treated with HNO_3 displayed a higher level of sugar release during enzymolysis than samples treated with either HCl or H_2SO_4. Taken in conjunction with the data showing hydrolysate sugar level following pre-treatment, this suggests that HNO_3 is the most effective acid of the three assessed, in the pre-treatment of SG. This is in agreement with other studies that have compared the efficiency of HNO_3 with a variety of other acids, in the pre-treatment of lignocellulose (White *et al*., 2008; Zhang *et al*., 2011).

It is noteworthy that hydrolysates derived from DSG contained higher levels of sugars than was observed in hydrolysates derived from BSG. This was the case following both pre-treatment and subsequent enzymolysis. This might be unexpected as the carbohydrate content of the grains documented in Chapter 3 showed that this particular source of maize based DSG had lower levels of extractable carbohydrate than BSG. However, as discussed in Chapter 3 the DSG documented here were from a grain distillery that employs a cooking process to the unmalted component of the mash prior to

mashing. This process is essentially a hot water pre-treatment and results in an increased extraction of sugars when compared to BSG that has not undergone a similar cooking process.

4.3.1.2 Acid concentration

As discussed in 4.3.1, out of the three acids utilised during this study it was found that HNO_3 performed best in terms of pre-treatment efficiency, both in being able to efficiently hydrolyse the hemicellulose fraction of SG and also to degrade lignin and decrease cellulose crystallinity thereby enabling further sugar extraction during enzymolysis. As such, further optimisation of conventional processes for the extraction of fermentable carbohydrate from SG, sought to assess the optimal Nitric acid concentration for pre-treatment.

Acid concentrations of 0.2, 0.5, 1 and 2 M were evaluated in terms of their ability to pre-treat DSG. Following pre-treatment, total sugar concentration within the hydrolysate (Fig. 4.5) varied depending upon the concentration of HNO_3 utilised during pre-treatment and this had an effect upon individual yields of glucose, arabinose and xylose (Table 4.5).

Table. 4.5 Effects of HNO3 concentration upon specific sugar release during pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis)

Acid concentration (M)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
0	0.12 (0.02)	4.11 (0.128)	4.23 (0.320)	8.46 (0.451)
0.2	1.02 (0.087)	6.63 (0.158)	8.98 (0.596)	16.63 (0.837)
0.5	3.29 (0.23)	6.46 (0.292)	15.6 (0.85)	25.35 (1.37)
1	4.06 (0.227)	7.53 (0.51)	16.41 (0.924)	28 (0.923)
2	4.34 (0.119)	4.06 (0.115)	10.2 (0.919)	18.6 (0.919)
Untreated DSG	0 (0)	0 (0)	0 (0)	0 (0)

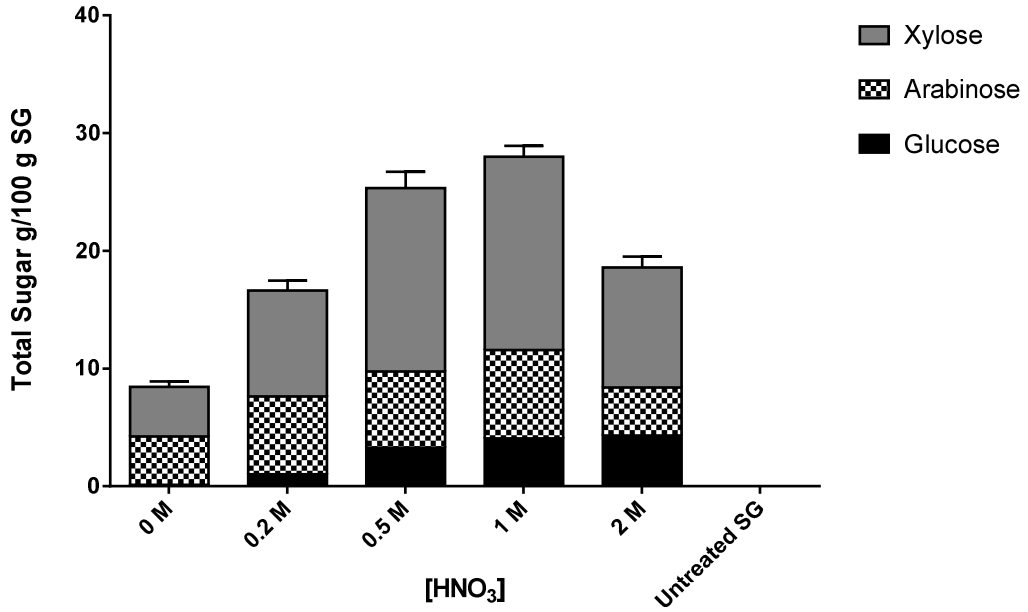


Fig. 4.5 Effects of HNO3 concentration upon sugar release during pre-treatment of DSG (Results are means of triplicate treatments with SD for total sugar)

Data from pre-treatment of DSG suggests that, up to a point, pre-treatment effectiveness rises with acid concentration, with 1 M HNO_3 being the most effective out of the four concentrations assessed. However, acid concentrations in excess of 1 M displayed less hydrolysate total sugar. Data from pre-treatment of DSG with 2 M HNO_3 displayed the highest hydrolysate glucose concentration, but lower levels of the pentose sugars arabinose and xylose. This suggests that 2 M HNO_3 was most effective in hydrolysing cellulose due to the high concentrations of glucose observed. This came at a cost as pentose levels were low resulting in a lower total hydrolysate sugars than observed in pre-treatment with 1M HNO_3. However, relatively high

levels of cellulose hydrolysis indicates that lignin and hemicellulose degradation is also high, but as pentose concentrations were low it suggests that there was a high level of pentose sugar degradation during the pre-treatment. Degradation of pentose sugars leads to the production of fermentation inhibitors. Xylose and arabinose can be degraded to furfural which can be further degraded to formic acid. Both of these compounds are extremely inhibitory to yeast fermentation and the acid pre-treatment employed during production of hydrolysates is likely to have generated both of these compounds (although they were not specifically analysed during this project).

Hydrolysate sugar levels following enzymolysis varied dependent upon the concentration of acid with which the DSG was pre-treated. The primary sugar release during enzymolysis was glucose with lower levels of the pentose sugars being released. This again indicates that the majority of SG hemicellulose hydrolysis occurs during acid pre-treatment rather than during enzymolysis. Again hydrolysate sugars increased with acid concentration up to a value to 1M HNO_3, with samples pre-treated with acid of this concentration displaying the highest total hydrolysate sugar following enzymolysis, as well as the greatest level of cellulose enzymolysis. Taking into account sugar data from both pre-treatment and enzymolysis, the data suggests that 1M HNO_3 is the most effective acid concentration for pre-treatment of the DSG used in this study.

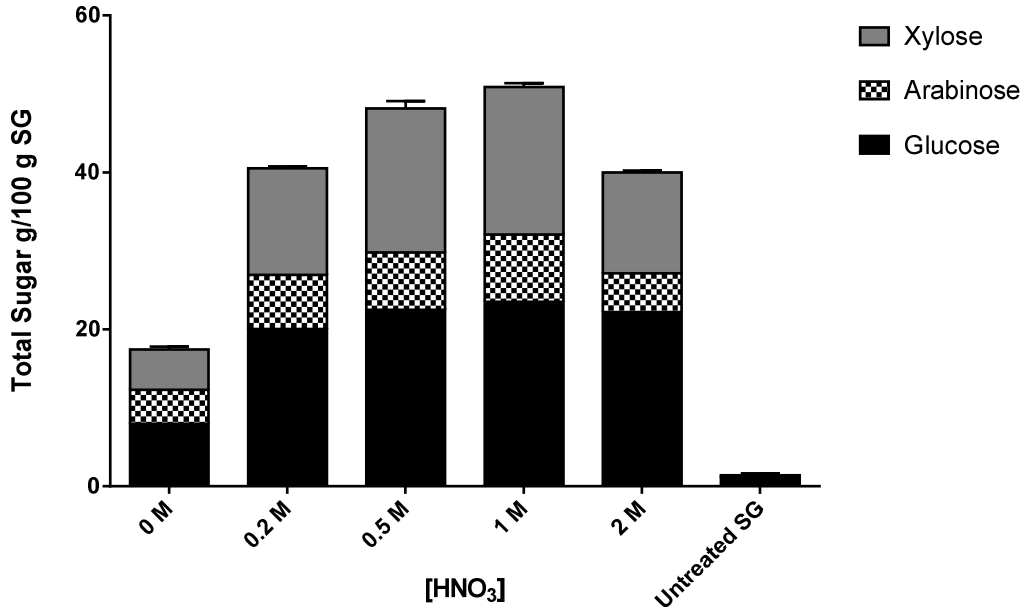


Fig. 4.6 Effects of the HNO3 pre-treatment concentration upon specific sugar release during enzymolysis of DSG (Results are means of triplicate treatments with SD for total sugar)

Table 4.6 Effects of the HNO3 pre-treatment concentration upon specific sugar release during enzymolysis of DSG (Results are means of triplicate treatments with SD in parenthesis)

Acid concentration (M)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
0	7.99 (0.142)	4.31 (0.103)	5.14 (0.087)	17.44 (0.361)
0.2	20.04 (0.052)	6.91 (0.061)	13.59 (0.197)	40.54 (0.238)
0.5	22.5 (0.18)	7.32 (0.357)	18.33 (0.910)	48.15 (0.910)
1	23.52 (0.116)	8.55 (0.131)	18.81 (0.416)	50.88 (0.476)
2	22.14 (1.014)	4.96 (0.18)	12.87 (0.238)	39.97 (0.238)
Untreated DSG	1.04 (0.123)	0.178 (0.067)	0.203 (0.049)	1.42 (0.214)

Investigations into the effects of HNO_3 concentration upon the efficiency of pre-treatment and enzymolysis of BSG, yielded results similar to those observed with DSG. Sugar release during pre-treatment showed an increase in hydrolysate sugars with increasing concentration up to a molarity of 1M, at which point increasing the acid concentration showed a reduction in hydrolysate sugars (Fig. 4.7). Hydrolysate sugars for SG treated with 2M HNO_3 showed high levels of glucose suggesting a relatively high level of cellulose hydrolysis (Table 4.7). However, apparent degradation of pentose sugars was observed causing a marked decrease in total hydrolysate sugar.

Table 4.7 Effects of HNO3 concentration upon specific sugar release during pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis)

Acid concentration (M)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
0	0.281 (0.034)	2.31 (0.096)	2.38 (0.099)	4.971 (0.117)
0.2	1.58 (0.097)	7.02 (0.009)	7.49 (0.208)	16.09 (0.208)
0.5	1.85 (0.103)	7.1 (0.094)	7.36 (0.157)	16.31 (0.364)
1	4.67 (0.139)	7.48 (0.148)	14.54 (0.271)	26.69 (0.486)
2	6.45 (0.109)	4.34 (0.065)	10.35 (0.027)	21.14 (0.270)
Untreated BSG	1.14 (0.106)	0.27 (0.067)	0.256 (0.051)	1.666 (0.174)

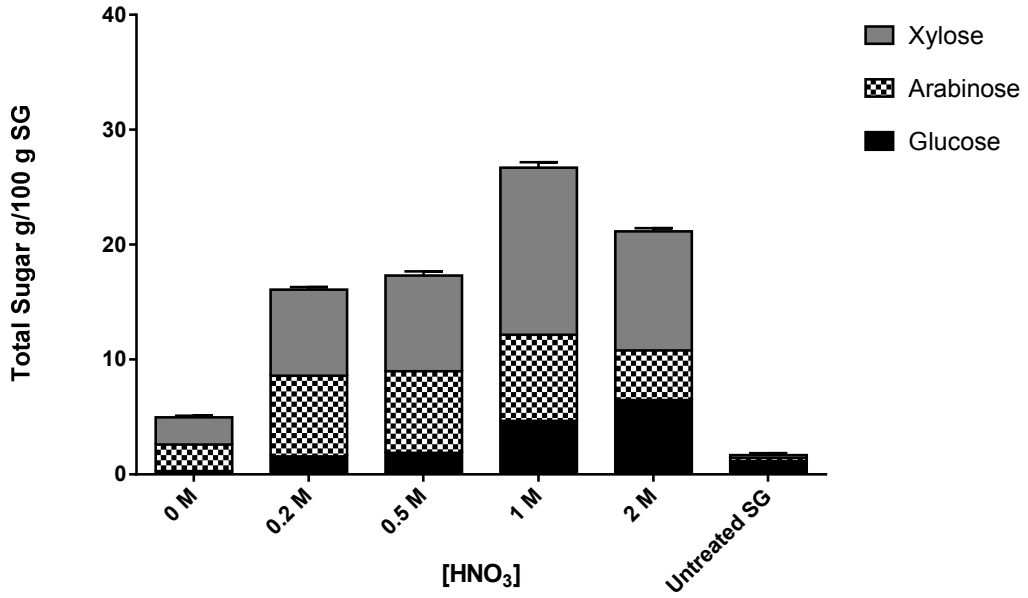


Fig. 4.7 Effects of HNO3 concentration upon sugar release during pre-treatment of BSG (Results are means of triplicate treatments with SD for total sugar)

Hydrolysate sugar levels increased significantly during enzymolysis of BSG and as previously observed the level of enzymatic sugar released was dependent upon the concentration of the HNO_3 used during pre-treatment (Fig. 4.8). 1M HNO_3 showed an increase total sugar yield compared to the other acid concentrations assessed and significantly more than the control sample exposed to heat treatment. This translated to an increase in the release of glucose, arabinose and xylose (Table 4.8). There was limited pentose sugar release during enzymolysis, with the majority of sugars hydrolysed comprising glucose. This supports the idea explored earlier, in that the majority of hemicellulose hydrolysis occurs during pre-treatment. Pre-treatment of BSG with HNO_3 in excess of 1M led to a reduced yield of hydrolysate sugars. This was evident after both pre-treatment and

enzymolysis and supports the notion already discussed, in that exposure of SG to high temperatures in combination with higher concentrations of nitric acid leads to sugar degradation.

Taking into account pre-treatment and enzymolysis data for both BSG and DSG, it is clear that treatment with 1M HNO_3 was the most effective acid concentration in terms of maximising hydrolysate sugars following both pre-treatment and enzymolysis. The results reported here are similar to results reported in the primary literature, in that increasing nitric acid concentration increases the sugars released during pre-treatment. White et al., (2008) reported that increasing the concentration of nitric acid from 0.08 to 0.16N resulted in an increase in hydrolysate sugar. However the researchers did not investigate the effects of acid concentrations in excess of 0.16N and as such did not observe sugar degradation caused by an acid concentration in excess of 1M reported here. Mussato and Roberto, (2005) reported that increasing sulphuric acid concentration from ~1 to 1.5 M resulted in a decrease in the concentration of pentose sugars as was observed in this study.

As was observed in previous experiments, DSG hydrolysates contained higher levels of sugars compared to BSG hydrolysates. This is in contrast to the total carbohydrate content of DSG and BSG detailed in chapter 3, where BSG was shown to have higher levels of extractable carbohydrate. However as discussed previously this is likely to be attributable to the fact the DSG used here was a maize based variety from a grain distillery which has undergone cooking prior to mashing. This essentially represents a prior pre-treatment and is likely to result in the

carbohydrate component of DSG being more easily extractable than carbohydrate in BSG.

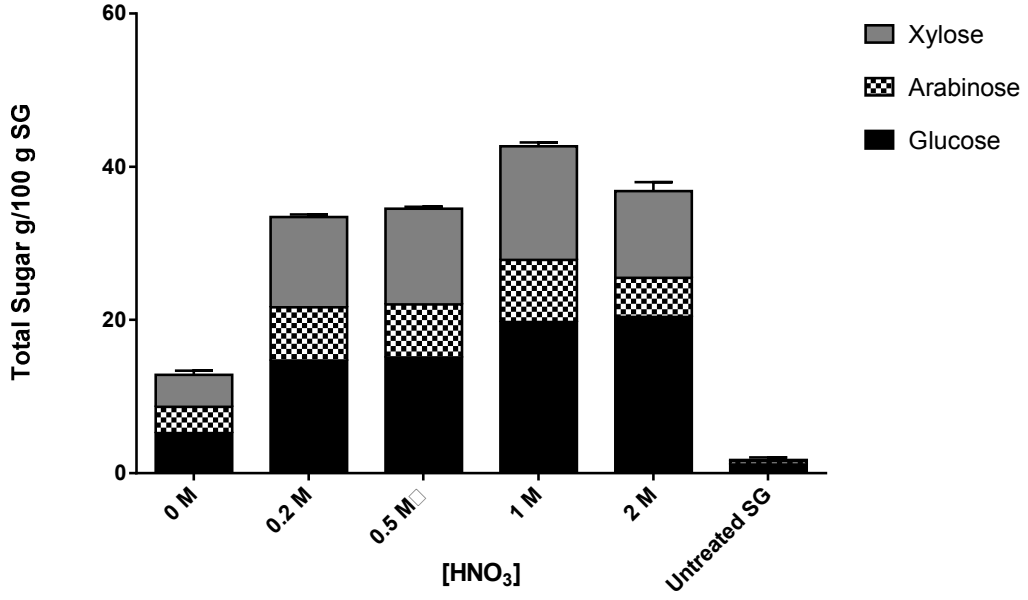


Fig. 4.8 Effects of the HNO3 pre-treatment concentration upon specific sugar release during enzymolysis of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 4.8 Effects of HNO3 concentration upon specific sugar release during enzymolysis of BSG (Results are means of triplicate treatments with SD in parenthesis)

Acid concentration (M)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
0	5.21 (0.187)	3.42 (0.116)	4.22 (0.274)	12.85 (0.502)
0.2	14.7 (0.137)	6.96 (0.101)	11.79 (0.09)	33.45 (0.323)
0.5	19.09 (0.274)	6.94 (0.122)	12.51 (0.27)	38.54 (0.270)
1	19.71 (0.392)	8.13 (0.027)	14.85 (0.167)	42.69 (0.476)
2	20.4 (0.207)	5.08 (0.351)	11.34 (0.63)	36.82 (1.180)
Untreated BSG	1.15 (0.136)	0.41 (0.067)	0.19 (0.073)	1.75 (0.311)

4.3.1.3 Temperature and duration of thermal decomposition

Research during this project, that sought to optimise the use of acid and heat in the pre-treatment and enzymatic digestion of SG, had thus far established the most effective acid in terms of species and concentration (i.e. 1M HNO_3). Further research sought to assess the effects of temperature and residence time in the pre-treatment of SG with 1M HNO_3. This was achieved by assessing hydrolysate sugars following both pre-treatment and enzymolysis at a variety of temperature and residence times.

In terms of DSG, there was a marked variation in hydrolysate sugars following pre-treatment, dependent on the temperature and residence time to which they had been exposed (Fig. 4.9). In general terms, as temperature and residence time rose, so did the level of hydrolysate sugars. However this was only observed up to a limit of pre-treatment parameters of 140℃ and a residence time of 20 min, after which hydrolysate sugars started to decrease. It is however noteworthy that as temperature and residence time rose, glucose concentrations increased. This indicates that increasing temperature and/or residence time increases the level of cellulose hydrolysis during pre-treatment.

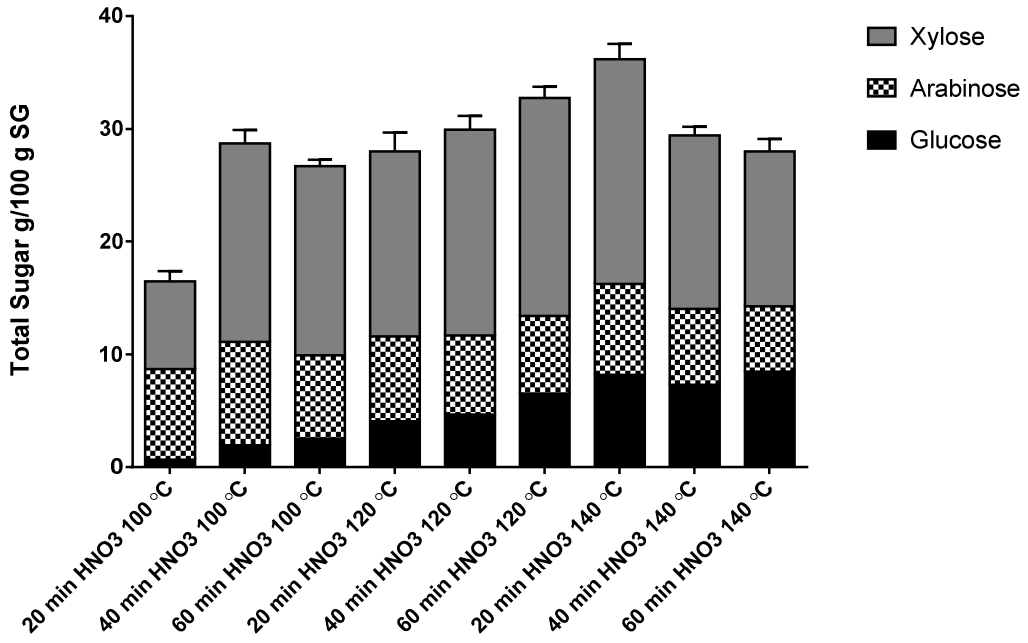


Fig. 4.9 Effects of temperature and residence time upon sugar release during HNO_3 pre-treatment of DSG (Results are means of triplicate treatments with SD for total sugar)

Following enzymolysis of pre-treated DSG, hydrolysate sugar concentrations increased as expected. However, total hydrolysate sugar concentrations varied dependent on the process parameters applied during pre-treatment (Fig. 4.10). Of the temperatures and residence times assessed, treatment at 120℃ with a residence time of 20 min, yielded the highest hydrolysate sugar concentration following enzymolysis. This is surprising as treatment with these parameters did not display the highest hydrolysate sugars following pre-treatment. It is likely that this can be attributed to the fact that pre-treatment at a higher temperature and/or residence time or lower temperature and higher residence time leads to a significant loss of hydrolysate sugar through acid degradation.

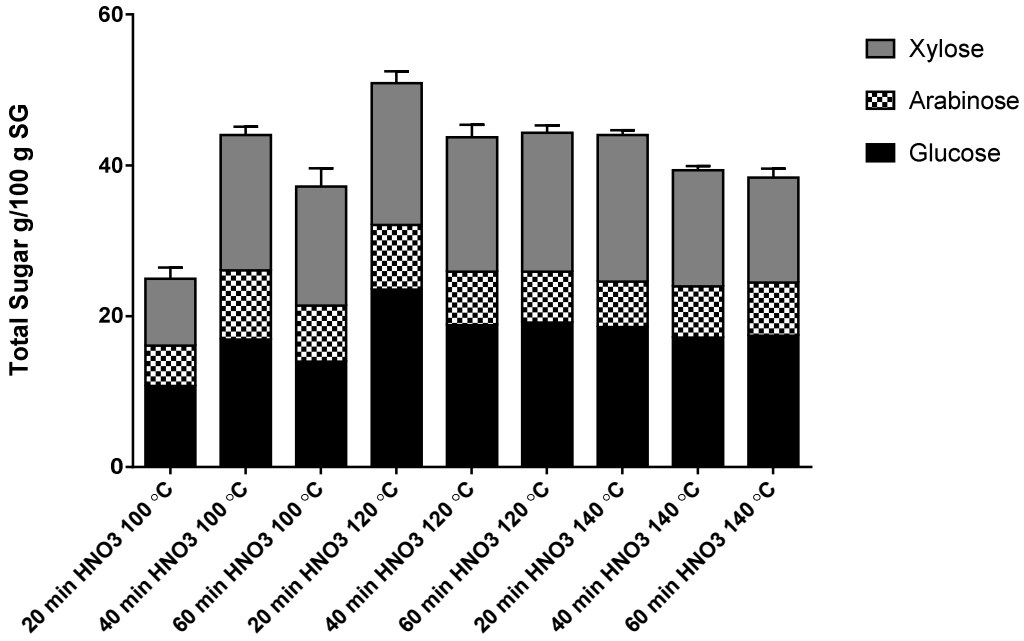


Fig. 4.10 Effects of the temperature and residence time applied during HNO_3 pre-treatment upon sugar release during enzymolysis of DSG (Results are means of triplicate treatments with SD for total sugar)

As was the case with DSG, there was a variation in BSG hydrolysate sugars dependent upon the temperature and residence time that was applied during pre-treatment. Whilst hydrolysate sugars increased with temperature and residence time, the increase was lower than that observed with DSG (Fig. 4.11). Following pre-treatment, parameters of 140℃ and residence time of 20 min showed the highest concentration of hydrolysate sugars. However this did not translate to the highest level of sugars following enzymolysis, which was observed in samples treated with parameters of 120℃ and 20 min (Fig. 4.12). As already discussed this is likely to be attributable to sugar loss through degradation during pre-treatment. Taking into account data from both BSG and DSG is it clear that pre-treatment parameters of 120℃ and residence time 20 min, was the most effective for DSG and BSG.

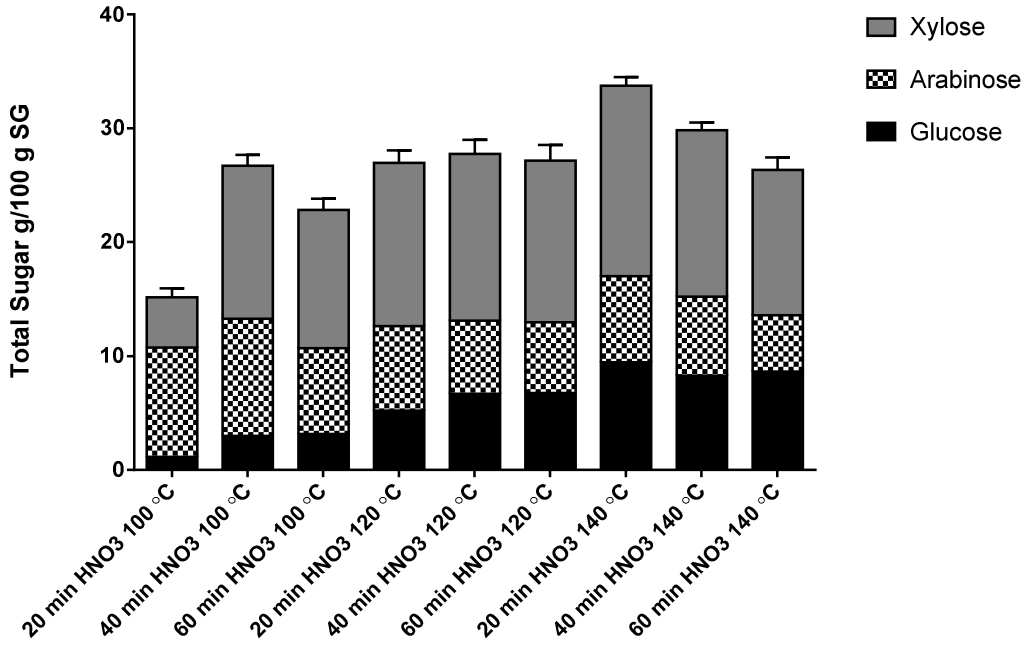


Fig. 4.11 Effects of temperature and residence time upon sugar release during HNO_3 pre-treatment of BSG (Results are means of triplicate treatments with SD for total sugar)

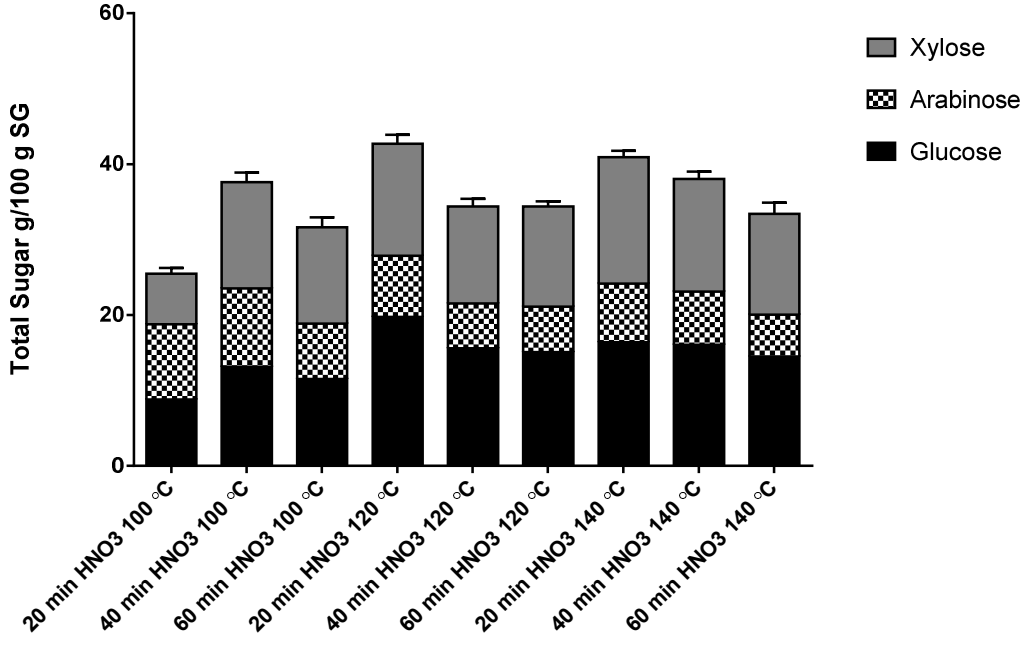


Fig. 4.12 Effects of the temperature and residence time applied during HNO_3 pre-treatment upon sugar release during enzymolysis of BSG (Results are means of triplicate treatments with SD for total sugar)

Heat degradation of hydrolysate sugars during pre-treatment can lead to the formation of a number compounds that are inhibitory to yeast in the subsequent fermentation. In the case of spent grains, hemicellulose hydrolysis yields xylose, arabinose, glucose and acetic acid, whilst cellulose hydrolysis yields glucose. At high temperatures the pentose sugars (xylose and arabinose) degrade to furfural with glucose degrading to form 5-hydroxymethy furfural (HMF) (Palmqvist and Hahn – Hagerdal, 1999). These compounds can further degrade, with formic acid being produced from HMF and furfural degradation. Additionally HMF can also form levulinic acid. Heat degradation of lignin yields a variety of phenolic compounds. Whilst not conducted during this study further insight into the levels of sugar degradation observed in samples pre-treated at 140℃ could have been achieved by quantifying sugar degradation products (furfural, HMF, formic and levulinic acid) that are inhibitory to fermentation by HPLC (Jonsson, Alriksson and Nilvebrant, 2013).

Results from both BSG and DSG suggest that pre-treatment with parameters of 140℃ and 20 minutes yielded the highest level of hydrolysate sugars. However this did not translate to a higher yield of sugars following enzymolysis with treatment for 20 minutes at 120℃ showing the highest sugar levels. As such it appears likely that whilst pre-treatment at 140℃ caused a higher degree of cellulose and hemicellulose hydrolysis, a degree of these sugars were then lost to heat degradation. This resulted in a decreased sugar yield following enzymolysis compared to other pre-treatment parameters due to there being less intact cellulose and hemicellulose to be enzymatically digested.

It is clear that as pre-treatment residence time rose above 20 minutes at 140℃ there was a marked reduction in the levels of hydrolysate pentose sugars. However there was still a relatively high level of glucose present within hydrolysates. Lignin and hemicellulose act as barrier to cellulose hydrolysis with both components surrounding the cellulose fibre. As such both lignin and hemicellulose must be degraded to render cellulose amenable to hydrolysis. The glucose levels within hydrolysates pre-treated in excess of 20 minutes at 140℃ suggests at least partial cellulose degradation however the levels of hydrolysate pentose sugars were lower than samples pre-treated at lower temperatures. As hemicellulose must be degraded before cellulose hydrolysis can occur it appears likely that the low levels of hydrolysate pentose sugars can also be attributed to heat degradation. This phenomenon has been reported by other researchers seeking to optimise acid pre-treatment of SG (Carvalheiro *et al*., 2004) and further insight could be gained into the levels of sugar degradation reported here by HPLC characterisation of sugar degradation products such as furfural, HMF, formic and levulinic acid.

4.3.2 Variables effecting carbohydrate release during enzymolysis - enzyme dosing and duration of enzymolysis

One of the major factors for successful commercialisation of cellulosic ethanol is the minimisation of the costs associated with the utilisation of commercially available cellulolytic enzymes. It is important that enzyme dosing is optimised so that enzyme dosing is minimal. However, enzyme dosing is a trade off between minimising dosing and ensuring that dosing still

enables efficient cellulolysis. Consequently, further optimisation of conventional methods for the pre-treatment and enzymolysis of SG, sought to assess the optimal dosing levels of the enzymes used in this research (Cellic Ctec and Htec, Novozymes, Denmark). This was achieved by pre-treating SG using the previously optimised methodology, namely, the use of 1M HNO3 with thermal treatment parameters of 120 ℃ for 20 min. Pre-treated material was then dosed with cellulolytic enzymes, at various dosing levels and efficiency of enzymolysis was assessed by characterising the sugars within the hydrolysate, at varying time points. In order to assess only the sugars released during enzymolysis sugar data was corrected to remove sugar release during pre-treatment.

In terms of total sugar content during enzymolysis of DSG (Fig. 4.13) there was little difference in rate of enzymolysis between samples dosed at 600 EGU/100 FXU and 1200 EGU/200FXU, this was observed in terms of total sugar release and specific assessment of glucose (Fig. 4.14), xylose (Fig. 4.15) and arabinose (Fig. 4.16) release. Both enzyme dosing levels showed maximum hydrolysis of sugars by the 24 h time point, after which there was minimal increase in enzymatic sugar yields was observed with increasing incubation time. As would be expected, the sample dosed with the lowest level of both enzymes showed the slowest rate of enzymatic sugar release and total hydrolysate sugar continued to increase throughout the observation period. Samples dosed with the lowest level of enzyme had still not reached the 24 h maximum achieved by the two higher dosing levels at 96 h.

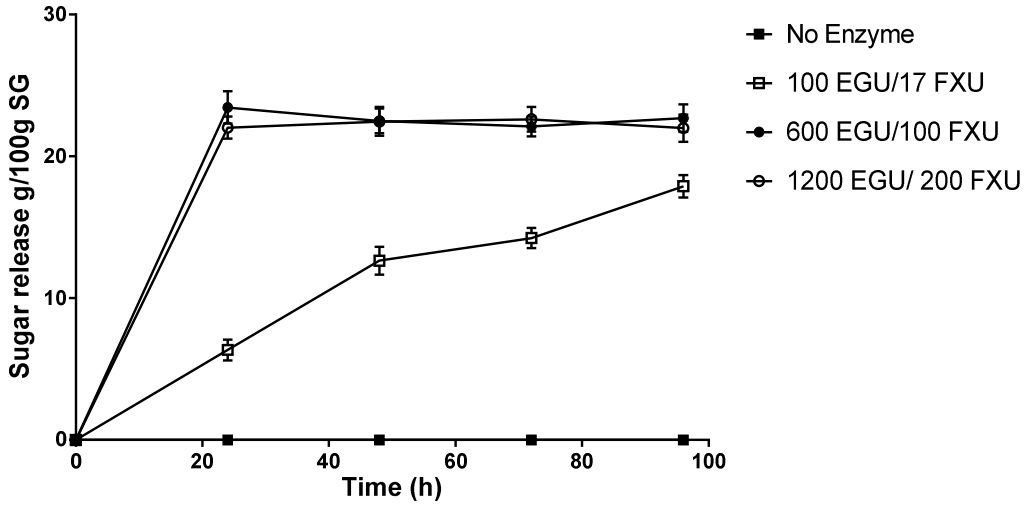


Fig. 4.13 Effects of enzyme loading upon total sugar release during enzymolysis of DSG (Results are means of triplicate treatments with error bars showing SD)

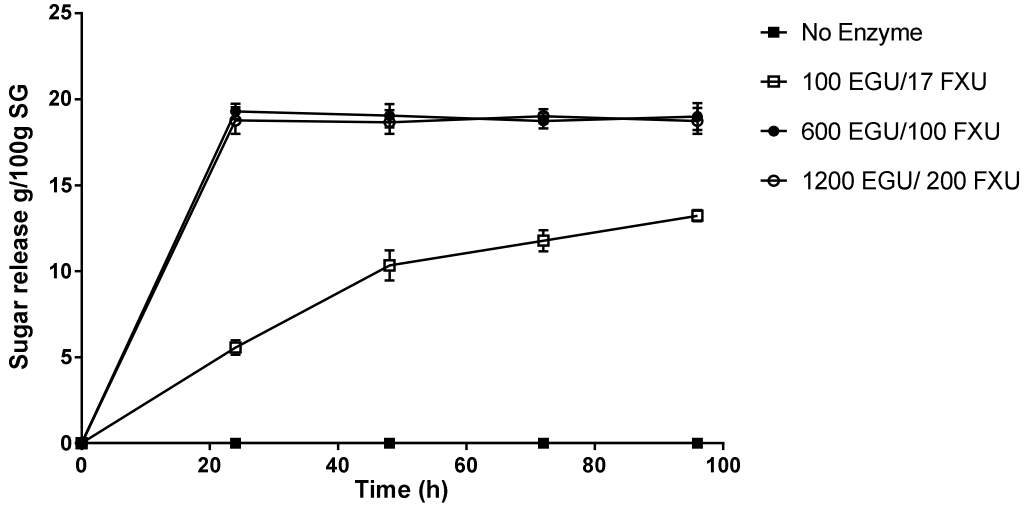


Fig. 4.14 Effects of enzyme loading upon glucose release during enzymolysis of DSG (Results are means of triplicate treatments with error bars showing SD)

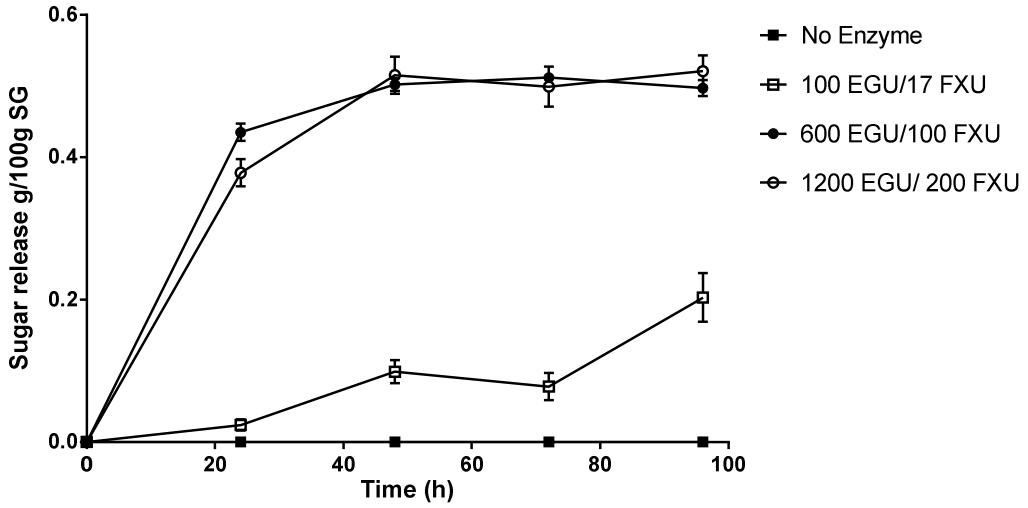


Fig. 4.15 Effects of enzyme loading upon arabinose release during enzymolysis of DSG (Results are means of triplicate treatments with error bars showing SD)

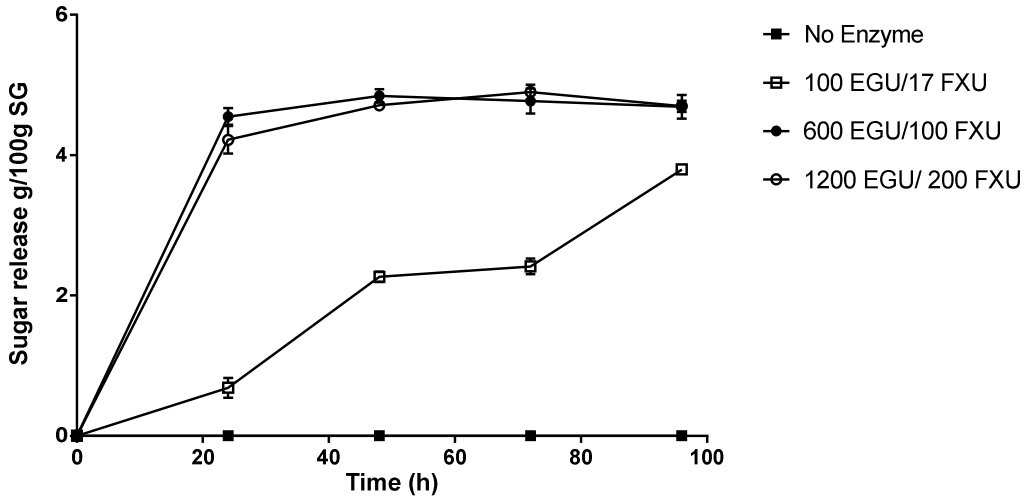


Fig. 4.16 Effects of enzyme loading upon xylose release during enzymolysis of DSG (Results are means of triplicate treatments with error bars showing SD)

Data relating to enzymolysis of BSG, was broadly similar to that obtained from DSG. Both of the higher enzyme dosing levels (600 EGU/100 FXU and 1200 ECU and 200 FXU) displayed no significant difference in rate of enzymatic sugar release, with both reaching maximum yield by 24 h. This was the case for total carbohydrate (Fig. 4.17) and individual levels of glucose (Fig. 4.18), arabinose (Fig. 4.19) and xylose (Fig.4.20). The sample dosed with the lowest of the enzyme dosing parameters displayed a gradual increase in hydrolysate sugars throughout the 96 h incubation period and just reached the maximum yield observed with the two higher dosing levels by this time point. Whilst feasible at the lab scale, a 96h incubation time would be undesirable within an industrial context. As such the higher enzyme dose would be more applicable in industry.

It was noteworthy that all three dosing levels showed minimal levels of pentose release during the enzymolysis period, with the majority of the sugars hydrolysed during the digestion being glucose. This confirms the point discussed earlier, that acid pre-treatment of SG hydrolyses the majority of the hemicellulose during the pre-treatment stage. Taking into account the data from both DSG and BSG it is clear that the optimum enzyme dosing level, out of the three assessed, was Ctec 600 EGU and Htec 100 FXU. These findings laid the foundation for further studies (described in Chapter 6) that aimed to evaluate the effects of ultrasound upon the activity of the enzymes involved in the enzymolysis of lignocellulose.

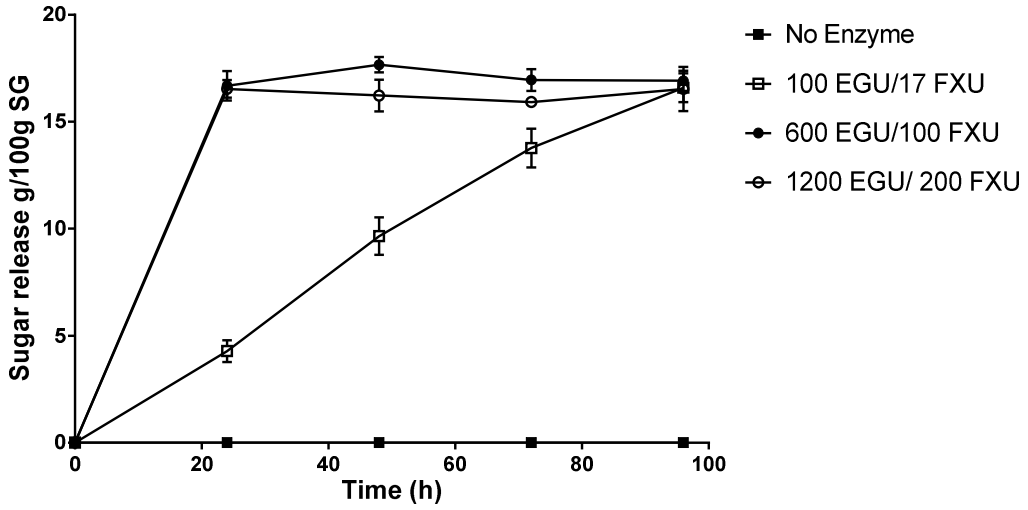


Fig. 4.17 Effects of enzyme loading upon total sugar release during enzymolysis of BSG (Results are means of triplicate treatments with error bars showing SD)

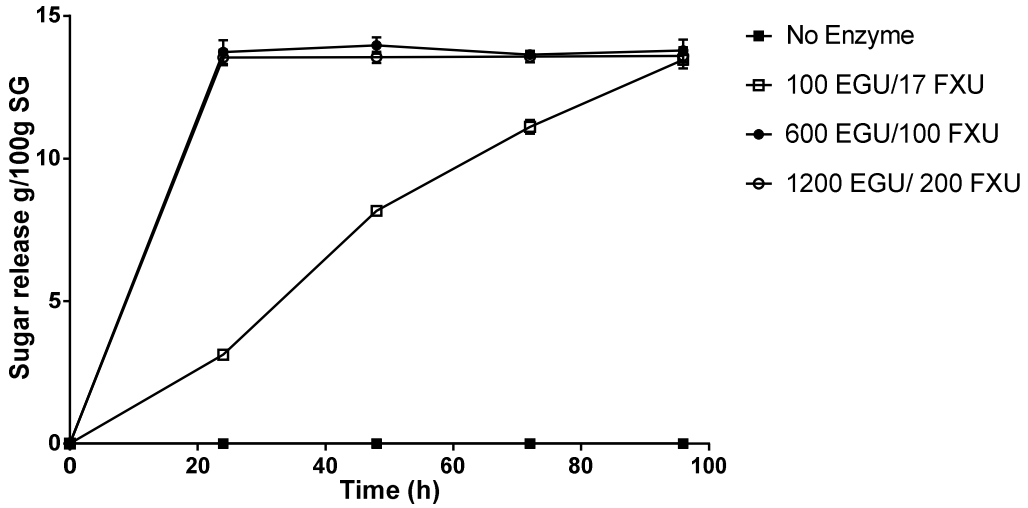


Fig. 4.18 Effects of enzyme loading upon glucose release during enzymolysis of BSG (Results are means of triplicate treatments with error bars showing SD)

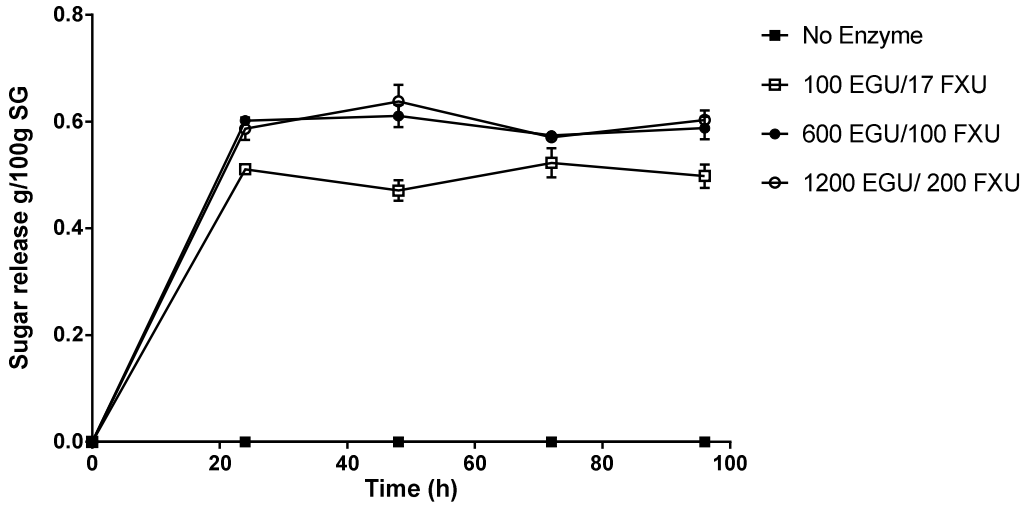


Fig. 4.19 Effects of enzyme loading upon arabinose release during enzymolysis of BSG (Results are means of triplicate treatments with error bars showing SD)

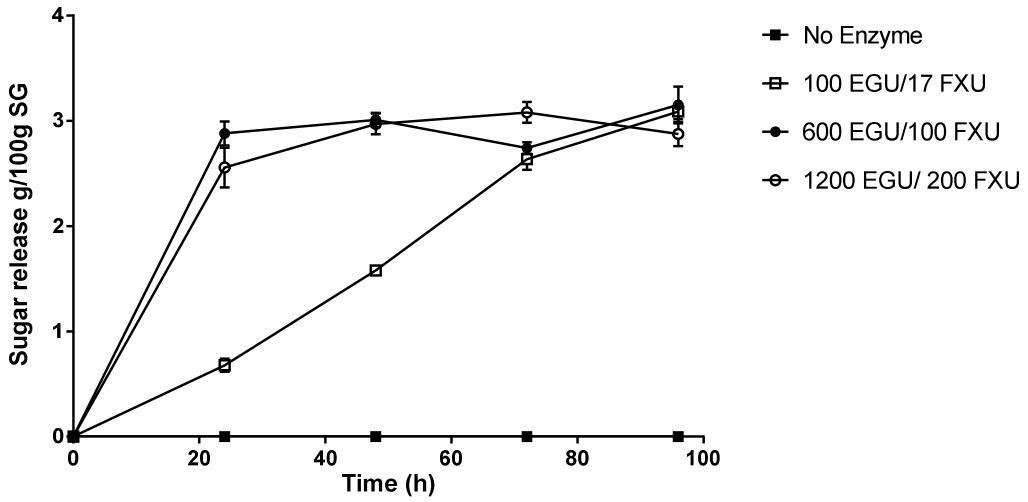


Fig. 4.20 Effects of enzyme loading upon xylose release during enzymolysis of BSG (Results are means of triplicate treatments with error bars showing SD)

4.4 Optimised methodology for the extraction of carbohydrate using classical techniques

Research looking to optimise pre-treatment and enzymolysis parameters, in terms of enzymatic digestion of SG, concluded that the optimal regime was treatment with 1M HNO_3, with thermal treatment for 20 min at 120℃ and enzyme dosing of Ctec at 600 EGU and HTec 100 FXU, with an incubation period of 24 h. Table 4.1 shows the maximum extractable carbohydrate achieved with these pre-treatment parameters, actual carbohydrate content and percentage conversion of potentially extractable carbohydrate. Conversion efficiencies from the use of acid in the pre-treatment and enzymolysis of SG, were used as a bench-mark with which to compare the ultrasonic pre-treatment techniques that are detailed in Chapter 5.

Table 4.9 – Conversion efficiency of optimised conventional hydrolysis of SG

SG type	Total carbohydrate content (g/100g SG)	Actual carbohydrate extracted (g/100g SG)	% Conversion
DSG	56.75	50.88	89.66
BSG	51.36	42.69	83.12

4.5 Summary

- The use of acid is highly effective in the pre-treatment and enzymolysis of SG.

- Type of acid, concentration, temperature and duration of heat treatment and enzyme load and duration of enzymolysis are all important in determining the efficiency of the extraction of carbohydrates from SG

- Optimised process parameters for the pre-treatment and enzymolysis of SG are: treatment with 1M HNO_3 at 120℃ for 20 min, followed by enzyme loading of Ctec 600 EGU and Htec 100 FXU and an incubation period of 24h.

Chapter 5

The use of ultrasound in the pre-treatment of spent grains

5. The use of ultrasound in the pre-treatment of spent grains

5.1 Introduction

5.1.1 Ultrasound induced acoustic cavitation and the pre-treatment of lignocellulose

When ultrasound is applied to a liquid medium a phenomenon referred to as cavitation occurs. Cavitation is defined as the formation, expansion and collapse of gaseous bubbles due to the application of ultrasound (Liang *et al*., 2006). The bubbles oscillate between growth and collapse through compression and rarefaction (Joseph *et al*., 2000, Peller *et al*., 2001). The collapse of micro-bubbles produced by ultrasound causes localised increases in temperature to between 3000-5000K and pressures of up to 10,000 atm. Additionally the collapse of cavitation bubbles causes the release of a high pressure jet of liquid as the bubble implodes. Other than the physical effects of cavitation bubble implosion ultrasound produces chemical effects through the dissociation of water to form hydroxyl and hydrogen radicals (Joseph *et al*., 2000).

As discussed in chapter 1, the use of ultrasound as a lignocellulose pre-treatment technology is a research field that is gaining increasing interest as in theory the action of ultrasonically induced cavitation is likely to lead to the degradation of biomass that is exposed to ultrasound. In addition to this hydroxyl radical production by ultrasound is likely to have an oxidising and possibly a hydrolytic effect upon the biomass. There are relatively few studies that have sought to assess the pre-treatment of lignocellulose with solely ultrasound, with the majority of recent publications seeking to determine if ultrasound can augment already established chemical methods.

These usually document that ultrasound increases the effectiveness of alkaline pre-treatment (Hromadkova and Ebringerova, 1998; Hromadkova and Ebringerova, 2003; Hromadkova and Ebringerova, 2008; Yuan *et al*., 2010). However it has been reported as effective in the pre-treatment of rice hull (Yu *et al*., 2008) and sunflower husk (Sulman *et al*., 2011). Yu *et al*., (2008) exposed rice hull to ultrasound at 50 kHz and 250 W and found that enzymatic digestion of treated samples resulted in significantly higher level of hydrolysate sugars compare to enzymatic digestion of untreated samples. Sulman *et al*., (2011) exposed sunflower husk to ultrasound at 30 kHz and reported that it was effective in rendering the polysaccharides within the biomass amenable to further hydrolysis.

5.1.2 Ozone, hydrogen peroxide, utrasound and advanced oxidation processes in the pre-treatment of lignocellulose

5.1.2.1 Ozone and hydrogen peroxide

There are a variety of studies that have shown that both hydrogen peroxide and ozone are effective in the pre-treatment of lignocellulose. Ozone is an unstable gas and is an extremely powerful oxidising agent. It has a high specificity towards reacting with compounds which contain high levels of double bonds (e.g. C=C, C=N, N=N) (García-Cubero *et al*., 2009). It has a lower potential to oxidise compounds containing single bonds. The use of ozone in the pre-treatment of lignocellulose is expanding and it has been shown to be effective in the pre-treatment of a wide variety of lignocellulosic biomass types with the mechanism by which it acts upon the biomass generally believed to be through oxidation and removal of lignin from the

lignocellulose structural matrix (Quesada *et al*., 1999; Silverstein *et al*., 2007; García-Cubero *et al*., 2009; Vidal and Molinier, 1988). Lignin surrounds the hemicellulose and cellulose component of the micro-fibril. As such its removal exposes cellulose and hemicellulose to further enzymatic hydrolysis.

Hydrogen peroxide is another oxidative chemical that has been shown to be effective in the pre-treatment of lignocellulose. Whilst it has a lower relative oxidation potential than ozone (Vogelpohl and Kim, 2004), it has been reported that it is effective in the oxidation of lignin and the partial hydrolysis of hemicellulose (Gould, 1985; Martel and Gould, 1990; Kim *et al*., 2001; Yang *et al*., 2002). As is the case with lignocellulose pre-treated with ozone, the removal of lignin exposes cellulose and hemicellulose so that they can be further hydrolysed.

5.1.2.2 Ultrasound and advanced oxidation processes

Whilst there is a relatively large volume of research documenting the use of either ozone or hydrogen peroxide in the pre-treatment of lignocellulose, there are at present no studies detailing the use of both compounds together. However the use of ozone and hydrogen peroxide is an established technique within the field of industrial wastewater treatment being one of a number of techniques defined as Advance Oxidation Processes (AOP). They are particularly effective in the degradation of phenol which is a common pollutant in wastewaters produced by industries such as petrochemicals, pharmaceuticals and organic chemical manufacture (Benitez *et al*., 1999). The use of AOP's is also an established method in the field of water disinfection and purification (Chin and Berube, 2005).

AOP's rely on the generation of hydroxyl radicals (HO●) which are characterised as being the neutral form of the hydroxide ion (OH^-) and are a type of short-lived reactive oxygen species (ROS) (Glaze *et al*., 1987). Whilst there are relatively few studies that document the use of AOP's and hydroxyl radicals in the pre-treatment of lignocellulose there are a number of studies that propose that a variety of fungal species employ free radicals in order to degrade lignocellulose.

It is now generally recognized that many species of wood decay basidiomycetes harness the power of hydroxyl radicals in the biodegradation of lignocellulose (Wood, 1994; Blanchette, 1995; Hyde and Wood, 1997; Hammel *et al*., 2002). It was once believed that wood decay fungi degrade lignocellulose via the secretion of a variety of hydrolytic enzymes including peroxidises, laccases and cellulases (Hammel *et al*., 2002). Whilst this is indeed the case, it is now known that lignocellulose degrading enzymes are too large to penetrate lignified cell walls in sound wood (Hammel *et al*., 2002) and this is further evidenced by the fact that lignocellulose residues require pre-treatment prior to enzymatic sugar extraction. Although not proven conclusively, it is now thought that wood decay fungi initiate wood decay through the production of ROS which attack the lignocellulose structural matrix in what is in effect a biologically employed lignocellulose pre-treatment mechanism. The general consensus within the primary literature is that wood decay fungi are most likely to produce hydroxyl radicals through the Fenton reaction (Eq. 1.1) (Backa *et al*., 1992; Jensen *et al*., 2001)

$$H_2O_2 + Fe^{2+} + H^+ \longrightarrow H_2O + Fe^{3+} + HO\bullet \quad (1.1)$$

Hydroxyl radicals have been shown to degrade lignin through the oxidation of C-H bonds contained within lignin subunits (Ek, Gierer and Jansbo, 1989). The actions of hydroxyl radicals upon the polysaccharides present within lignocellulose are less well understood, however it is thought that they degrade polysaccharides through the removal of hydrogen from sugar subunits (Hammel *et al*., 2002). The excretion of hydroxyl radicals by wood decay fungi ultimately leads to initiation of lignin degradation. This allows penetration by laccase enzymes which increase the rate at which lignin is degraded. The structure of the lignocellulose matrix, with lignin encasing the polysaccharides contained within cellulose and hemicellulose, dictates that once lignin is hydrolysed the polysaccharides are then exposed to further hydrolysis by the hydrolytic enzymes excreted by wood decay fungi.

Other than the Fenton reaction employed by various fungal species, a variety of AOP's exist and differ in the mechanism by which they generate hydroxyl radicals. They usually fall under two broad categories, those being either photochemical or non-photochemical systems. A number of non-photochemical AOP's exist including the use of ozone and hydrogen peroxide, ozone and activated carbon, electron beam irradiation and radiolysis (Gogate and Pandit, 2004). Photo-chemical AOP's usually involve the application of UV irradiation with one or more oxidants such as ozone and hydrogen peroxide (Gogate and Pandit, 2004).

The reaction of ozone and hydrogen peroxide (peroxone reaction) is one type of non-photochemical AOP that has shown to be effective in the production of hydroxyl radicals (Vogelpohl and Kim, 2004). The use of ozone and hydrogen peroxide has long been established in waste-water treatment (Camel and Bermond, 1998; Acero and von Guten, 2000) and the mechanisms of reaction are well understood and are summarized in Eq. 1.2 – 1.9 (Forni *et al*., 1982; Sehested *et* al., 1982; Buhler *et al*., 1984). Hydrogen peroxide can form the hydroperoxide ion and H^+ (Eq. 1.2). The hydroperoxide ion reacts with ozone to produce ozonide and the hydroperoxide radical (Eq. 1.3).

$$H2O2 \longrightarrow HO_2^- + H^+ \quad (1.2)$$

$$HO_2^- + O3 \longrightarrow O_3^- + HO_2\bullet \quad (1.3)$$

The products formed then enter into a chain reaction which results in the formation of hydroxyl radicals (Eq. 1.4-1.7).

$$HO_2^- \longrightarrow H+ + O_2^- \quad (1.4)$$

$$O_2^- + O_3 \longrightarrow O_3^- + O_2 \quad (1.5)$$

$$O_3^- + H+ \longrightarrow HO_3 \quad (1.6)$$

$$HO_3 \longrightarrow HO\bullet + O_2 \quad (1.7)$$

Following formation of hydroxyl radicals further generation is achieved via an autocatalytic reaction between ozone and the hydroxyl radical (Eq. 1.8-1.9).

$$O_3 + HO\bullet \longrightarrow O_2 + HO_2\bullet \quad (1.8)$$

$$O_3^- + HO_2\bullet \longrightarrow 2O_2 + HO\bullet \quad (1.9)$$

Photochemical AOP systems usually involve the use of UV irradiation in combination with one or more oxidative chemical species, such as ozone and/or hydrogen peroxide. The principal theory being that the action of UV irradiation upon H_2O_2 or O_3 enhances free radical formation through the dissociation of H_2O_2 or O_3 (Huang *et al.*, 1993). UV photolysis of H_2O_2 results in the production of hydroxyl radicals (HO●) (Eq. 1.9). UV photolysis of O_3 results in the formation of H_2O_2 (Eq. 1.10). The deprotonated species of hydrogen peroxide (HO_2^-) reacts with O_3 which results in the formation of ozonide (O_3^-) and subsequently hydroxyl radicals (HO●).

$$H_2O_2 + h\nu \longrightarrow 2\ HO\bullet \quad (1.9)$$

$$O_3 + h\nu \longrightarrow H_2O_2 \longrightarrow HO_2^- + O_3 \longrightarrow O_3^- \longrightarrow 2\ HO\bullet \quad (1.10)$$

Ultrasound can replace UV in causing the dissociation of ozone and/or hydrogen peroxide. The exact mechanism by which it acts upon ozone or hydrogen peroxide is unknown, however it is thought that it replaces the energy supplied by UV light with energy produced by collapse of cavitation bubbles (Gogate and Pandit, 2004). Thus, it is logical to suggest that ultrasound can enhance hydroxyl radical production in systems that use ozone and hydrogen peroxide in the generation of hydroxyl radicals. The rationale behind this suggestion is that ultrasound provides a more direct

route for the production of hydroxyl radicals (Eq. 1.9-1.10) than those observed in conventional peroxone AOP systems (Eq. 1.2-1.9).

5.2 Experimental approach

Whilst the use of ultrasound alone has been reported as effective in the pre-treatment of lignocellulose, the scarcity of publications in this area, suggests that it is an area which warrants further investigation. As has been discussed previously, hydroxyl radicals are deployed by various fungal species in the degradation of lignocellulose. Coupled with this ultrasound has been shown to generate hydroxyl radicals in aqueous systems through the homolysis of water. Additionally, ultrasound has been shown to generate hydroxyl radicals through interaction with ozone and/or hydrogen peroxide (Gogate and Pandit, 2004). As such it would appear that ultrasound has the potential to increase hydroxyl radical production in AOP systems that involve the use of hydrogen peroxide and ozone. However there is currently a lack of data regarding the use of ultrasound in combination with AOP's in the pre-treatment of lignocellulose. Consequently, research was conducted that sought to assess the use of ultrasound alone or in conjunction with oxidants in the pre-treatment of SG.

Of the four sources of spent grains assessed for compositional characterisation detailed in Chapter 3, two were selected for ultrasonic pre-treatment studies. The samples selected for study were spent grains from the grain distillery (referred to as DSG in subsequent data) which were derived from 85% maize/15% malted barley and those from Bellhaven Brewery (referred to as BSG in subsequent data) which were derived from

100% barley malt. The SG from the grain distillery was selected due to the fact they contain a large maize component, a feedstock which has particular relevance for the American bioethanol industry which currently has the largest capacity for bioethanol production globally. As such research which seeks to increase ethanol production from maize based feedstocks are of current topical importance. Samples of SG from the ale brewery were selected for logistical purposes in that the research group has large quantities available.

The use of ultrasound in the pre-treatment of SG was studied by exposing both BSG and DSG to ultrasound alone as well as in combination with acid, hydrogen peroxide and/or ozone and characterising the release of sugars both after pre-treatment and enzymolysis. Combined ultrasonic and chemical treatment was conducted by dosing the SG with acid or hydrogen peroxide within the reactor. Ozone work involved diffusing O_3 into the reactor during exposure of SG to ultrasound.

5.3 Results and Discussion

5.3.1 Ultrasonic pre-treatment of spent grains – 20 kHz

5.3.1.1 Influence of ultrasonic power upon 20 kHz ultrasound pre-treatment of SG

Preliminary experimentation sought to determine the effects of ultrasound, at various output powers, upon sugar release during pre-treatment and subsequent enzymolysis. Following exposure of DSG to ultrasound for 1 h (Fig. 5.1), there was an extremely low level of sugars released during pre-

treatment. This was seen in samples exposed to ultrasound as well as the non-sonicated control. This was also observed in BSG exposed to ultrasound for 1 h, with low levels of sugar released during treatment (Fig. 5.2) and little difference observed between samples treated with ultrasound and the control.

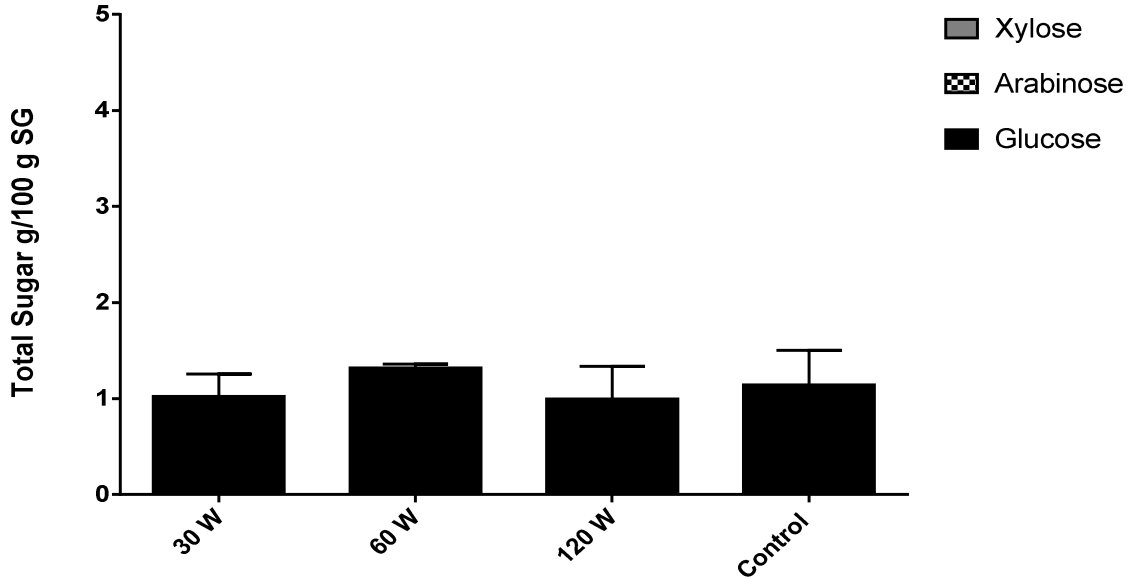


Fig. 5.1 Effects of ultrasonic output power upon sugar release following 20 kHz ultrasound treatment of DSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

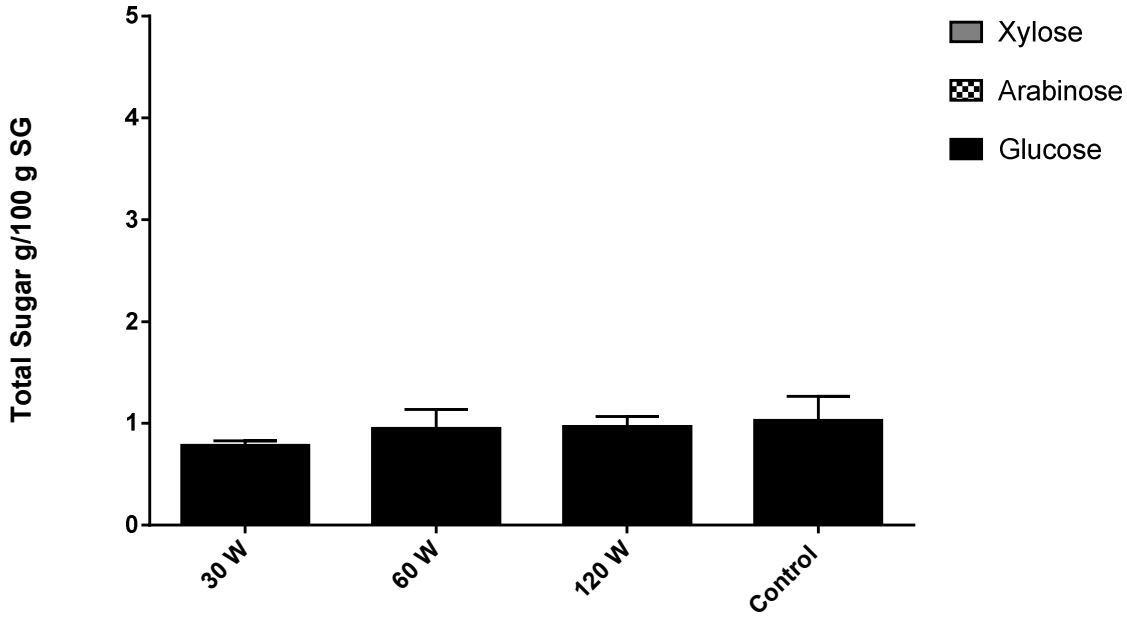


Fig. 5.2 Effects of ultrasonic output power upon sugar release following 20 kHz ultrasound treatment of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

In the case of both DSG (Table 5.1) and BSG (Table 5.2), samples that were exposed to ultrasound along with the control sample, the sugars found within the hydrolysate comprised solely glucose. As there was a lack of pentose sugars released this indicates that there was no hydrolysis of hemicellulose. This suggests that, rather than the glucose being obtained from cellulose or hemi-cellulose hydrolysis, it was the result of residual glucose left from the mashing process during the production of wort. It appears likely that the mechanical milling applied to the SG rendered residual glucose more amenable to water extraction.

Table 5.1 – Effects of ultrasonic power upon specific sugar release during 20 kHz ultrasound pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis)

Ultrasonic power (W)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
30	1.02 (0.234)	0 (0)	0 (0)	1.02 (0.234)
60	1.32 (0.038)	0 (0)	0 (0)	1.32 (0.038)
120	0.99 (0.341)	0 (0)	0 (0)	0.99 (0.341)
Control	1.15 (0.357)	0 (0)	0 (0)	1.15 (0.357)

Table 5.2 – Effects of ultrasonic power upon specific sugar release during 20 kHz ultrasound pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis)

Ultrasonic power (W)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
30	0.79 (0.045)	0 (0)	0 (0)	0.79 (0.045)
60	0.95 (0.187)	0 (0)	0 (0)	0.95 (0.187)
120	0.97 (0.097)	0 (0)	0 (0)	0.97 (0.097)
Control	1.03 (0.234)	0 (0)	0 (0)	1.03 (0.234)

Following the pre-treatment where samples were exposed to ultrasound, the SG and the control samples were incubated with a cocktail of hydrolytic enzymes that digest cellulose and hemicelluloses to free sugar. In the case of DSG, total hydrolysate sugar levels following enzymolysis were low (Fig. 5.3) and this was also observed after enzymolysis of BSG (Fig. 5.4). There was no significant difference in hydrolysate levels of glucose, arabinose and xylose between SG treated with ultrasound and the non-sonicated control. This was evident for DSG (Table 5.3) and BSG (Table 5.4).

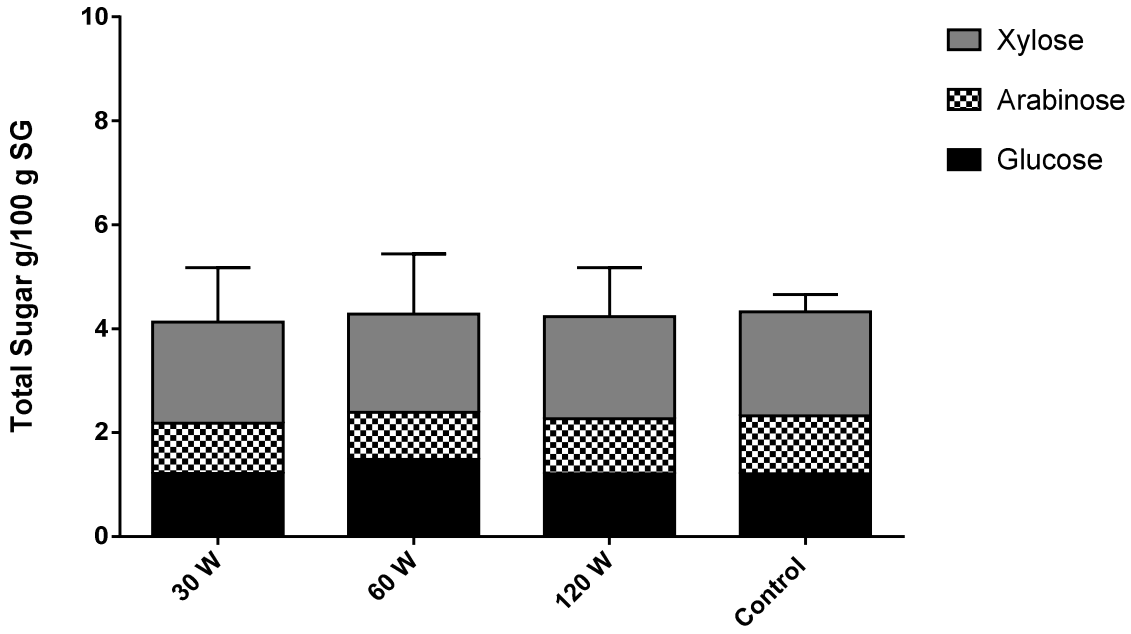


Fig. 5.3 Effects of ultrasonic output power upon sugar release during enzymolysis of DSG pre-treated with 20kHz ultrasound (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.3 Sugar release during enzymolysis of 20 kHZ ultrasound treated DSG – influence of ultrasonic power (Results are means of triplicate treatments with SD in parenthesis)

Ultrasonic power (W)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
30	1.21 (0.396)	0.97 (0.301)	1.95 (0.427)	4.13 (1.045)
60	1.49 (0.412)	0.89 (0.134)	1.89 (0.366)	4.27 (1.151)
120	1.21 (0.372)	1.06 (0.332)	1.96 (0.203)	4.23 (0.945)
Control	1.2 (0.098)	1.11 (0.116)	2.01 (0.178)	4.32 (0.333)

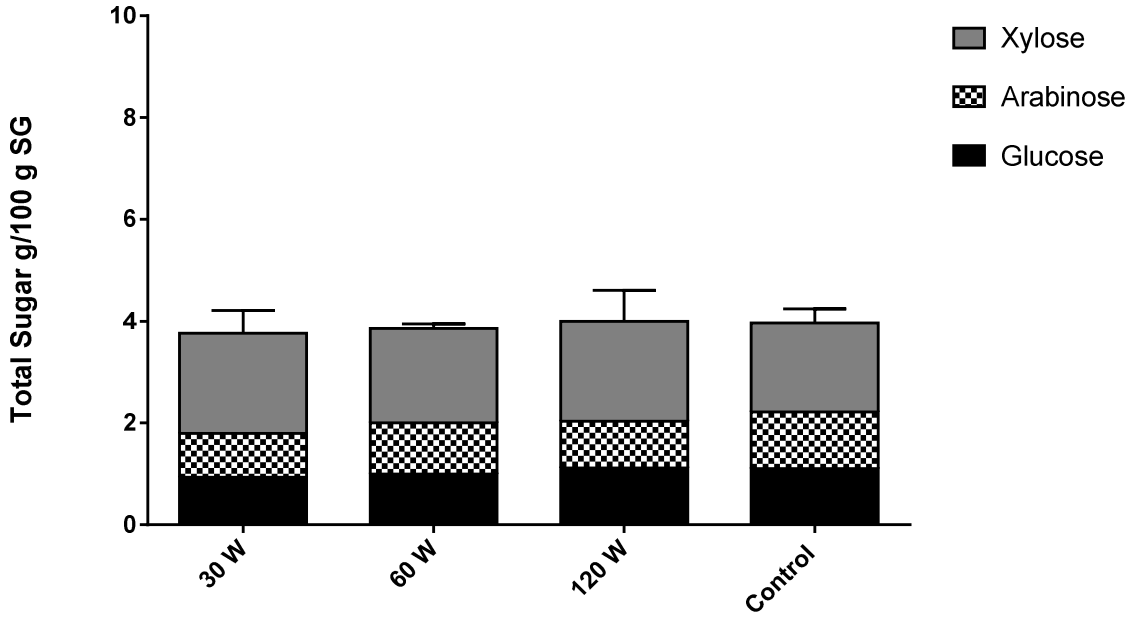


Fig. 5.4 Effects of ultrasonic output power upon sugar release during enzymolysis of BSG pre-treated with 20kHz ultrasound (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.4 Sugar release during enzymolysis of 20 kHZ ultrasound treated BSG – influence of ultrasonic power (Results are means of triplicate treatments with SD in parenthesis)

Ultrasonic power (W)	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
30	0.94 (0.061)	0.86 (0.126)	1.97 (0.267)	3.77 (0.451)
60	0.98 (0.017)	1.01 (0.032)	1.85 (0.048)	3.84 (0.089)
120	1.12 (0.189)	0.92 (0.093)	1.96 (0.361)	4 (0.612)
Control	1.09 (0.089)	1.11 (0.068)	1.75 (0.154)	3.95 (0.281)

It is clear that when taking into account data from exposure of DSG and BSG to ultrasound that, at the applied parameters of output wattage, ultrasound is not an effective technology in the pre-treatment of SG. Evidence for this relates to the fact that samples exposed to ultrasound showed no significant difference in hydrolysed sugars compared with the control samples. This was the case with sugar characterisation following both pre-treatment and enzymolysis. As discussed in Chapter 4, an effective pre-treatment technique results in lignocellulose being rendered amenable to enzymatic hydrolysis. Sugars released during enzyme treatment of samples exposed to ultrasound were extremely low compared to those released during a typical acid based pre-treatment outlined in Chapter 4 and similar to those released during enzymatic hydrolysis of the control sample.

5.3.1.2 Influence of ultrasonic residence time upon pre-treatment of SG

In order to further validate the theory that ultrasound alone is not a viable pre-treatment technique for SG, the effect of ultrasonic residence time was assessed in terms of its effect upon pre-treatment of lignocellulose. This was achieved by varying the time of ultrasonic irradiation and exposing the SG to the maximum ultrasonic power achievable by the apparatus (120 W). It was thought plausible that by increasing the residence time utilised during previous research that the use of ultrasound may be effective in lignocellulose pre-treatment.

HPLC hydrolysate sugar characterisation following exposure of DSG to ultrasound (Fig. 5.5), displayed extremely low levels of sugars released. This was similar to the levels of sugar release in the control samples. In the

case of sugar characterisation of BSG hydrolysates following exposure to ultrasound (Fig. 5.6), sugars released during pre-treatment were also low and not significantly different to the control samples. Hydrolysate sugars for samples exposed to ultrasound and the non-sonicated control, comprised solely glucose. This was observed for both DSG (Table 5.5) and BSG (Table 5.6) and again it appears likely that this glucose can be attributed to residual SG sugars, rather than hydrolysis of cellulose or hemicellulose.

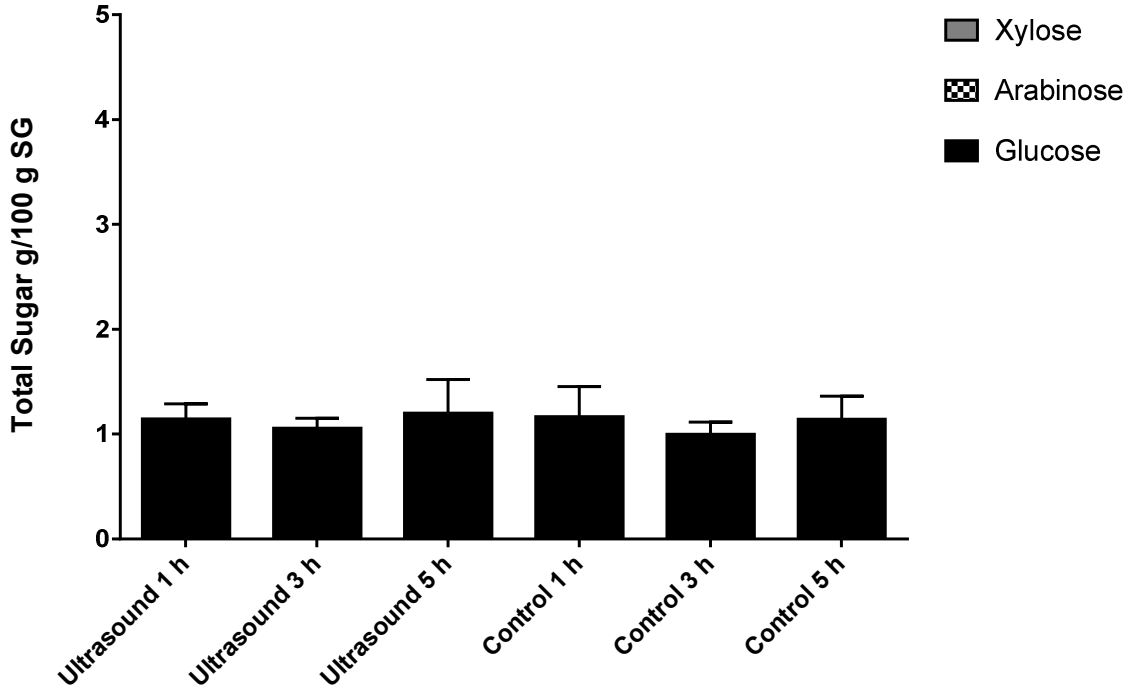


Fig. 5.5 Effects of residence time upon sugar release following 20 kHz ultrasound treatment of DSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

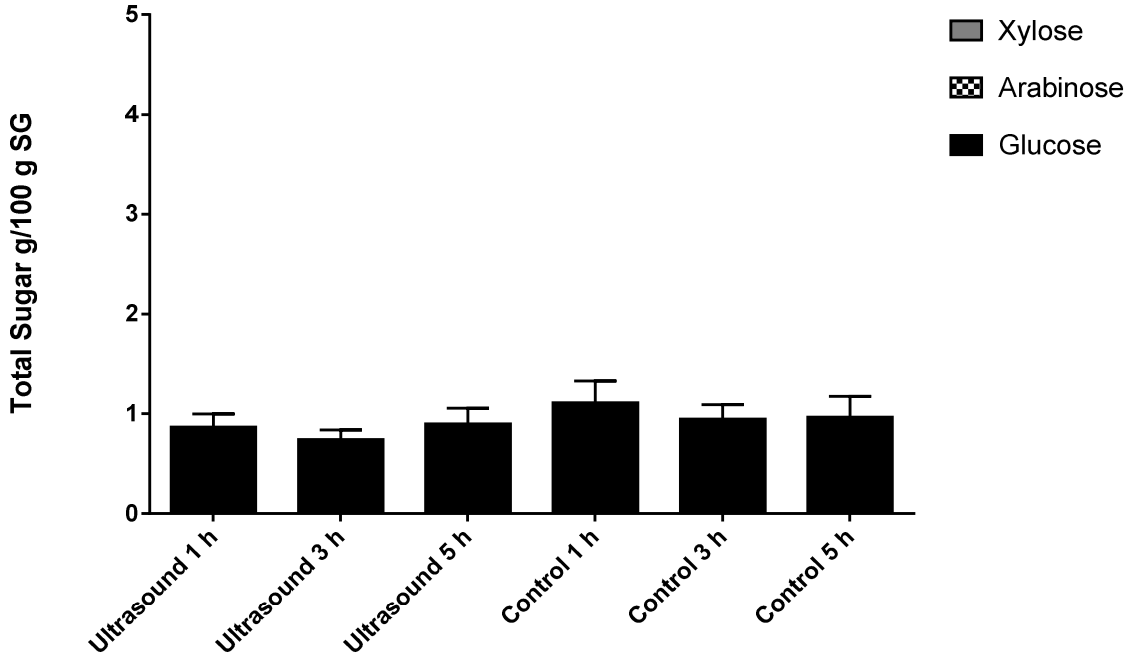


Fig. 5.6 Effects of residence time upon sugar release following 20 kHz ultrasound treatment of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.5 Effects of duration of ultrasonic pre-treatment upon specific sugars released from DSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound 1 h	1.15 (0.144)	0 (0)	0 (0)	1.15 (0.144)
Ultrasound 3 h	1.06 (0.096)	0 (0)	0 (0)	1.06 (0.096)
Ultrasound 5 h	1.2 (0.321)	0 (0)	0 (0)	1.2 (0.321)
Control 1 h	1.17 (0.284)	0 (0)	0 (0)	1.17 (0.284)
Control 3 h	0.99 (0.116)	0 (0)	0 (0)	0.99 (0.116)
Control 5 h	1.14 (0.222)	0 (0)	0 (0)	1.14 (0.222)

Table 5.6 – Effects of duration of ultrasonic pre-treatment upon specific sugars released from BSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound 1 h	0.87 (0.134)	0 (0)	0 (0)	0.87 (0.134)
Ultrasound 3 h	0.74 (0.097)	0 (0)	0 (0)	0.74 (0.097)
Ultrasound 5 h	0.9 (0.155)	0 (0)	0 (0)	0.9 (0.155)
Control 1 h	1.11 (0.219)	0 (0)	0 (0)	1.11 (0.219)
Control 3 h	0.95 (0.147)	0 (0)	0 (0)	0.95 (0.147)
Control 5 h	0.97 (0.207)	0 (0)	0 (0)	0.97 (0.207)

In order to further assess pre-treatment efficiency, samples exposed to ultrasound, along with the respective controls, were incubated with cellulolytic enzymes and the hydrolysate sugars were characterised after enzymolysis. In the case of ultrasonic treatment of DSG, total hydrolysate sugar levels did increase during enzymolysis (Fig. 5.7), however enzymatic sugar release was low. Low levels of enzymatic sugar release were also observed in the control samples. Glucose, arabinose and xylose release during enzymolysis of DSG, was similar to those observed during enzymolysis of the non-sonicated controls (Table 5.7).

Table 5.7 – Effects of duration of ultrasonic pre-treatment upon sugars released during enzymolysis of DSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound 1 h	1.21 (0.284)	1.21 (0.163)	2.12 (0.114)	4.54 (0.568)
Ultrasound 3 h	1.09 (0.073)	1.04 (0.064)	1.89 (0.091)	4.02 (0.177)
Ultrasound 5 h	1.26 (0.133)	1.11 (0.167)	2.1 (0.305)	4.47 (0.691)
Control 1 h	1.19 (0.020)	1.35 (0.150)	2.12 (0.242)	4.66 (0.333)
Control 3 h	1.02 (0.096)	1.09 (0.079)	1.97 (0.153)	4.08 (0.364)
Control 5 h	1.22 (0.130)	0.95 (0.060)	1.84 (0.123)	4.01 (0.214)

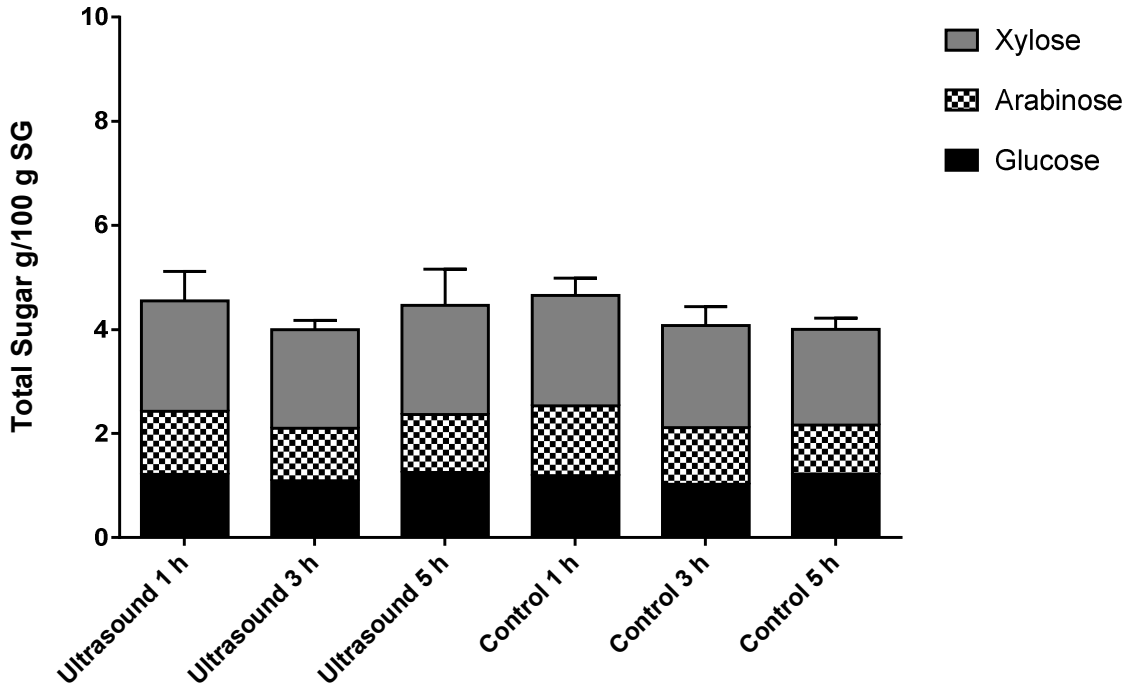


Fig. 5.7 Effects of residence time upon sugar release during enzymolysis of DSG pre-treated with 20kHz ultrasound (Results are means of triplicate treatments with error bars showing SD for total sugar)

During enzymolysis of BSG that had been treated with ultrasound, hydrolysate sugar levels increased (Fig. 5.8), however as was the case with DSG the sugars released during enzymolysis were low. There was a minor release of glucose, arabinose and xylose during enzymolysis (Table 5.8), but this was similar to sugar release during enzymolysis of the non-sonicated controls.

Table 5.8 Effects of duration of ultrasonic pre-treatment upon specific sugars released during enzymolysis of BSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound 1 h	0.96 (0.022)	0.87 (0.028)	1.79 (0.086)	3.62 (0.113)
Ultrasound 3 h	0.85 (0.046)	0.91 (0.105)	1.97 (0.167)	3.73 (0.274)
Ultrasound 5 h	0.94 (0.066)	0.9 (0.026)	1.63 (0.043)	3.47 (0.099)
Control 1 h	1.16 (0.030)	1.06 (0.045)	2.01 (0.133)	4.23 (0.187)
Control 3 h	1.06 (0.185)	0.97 (0.067)	1.91 (0.128)	3.94 (0.301)
Control 5 h	1.13 (0.104)	1.27 (0.397)	1.95 (0.189	4.35 (0.711)

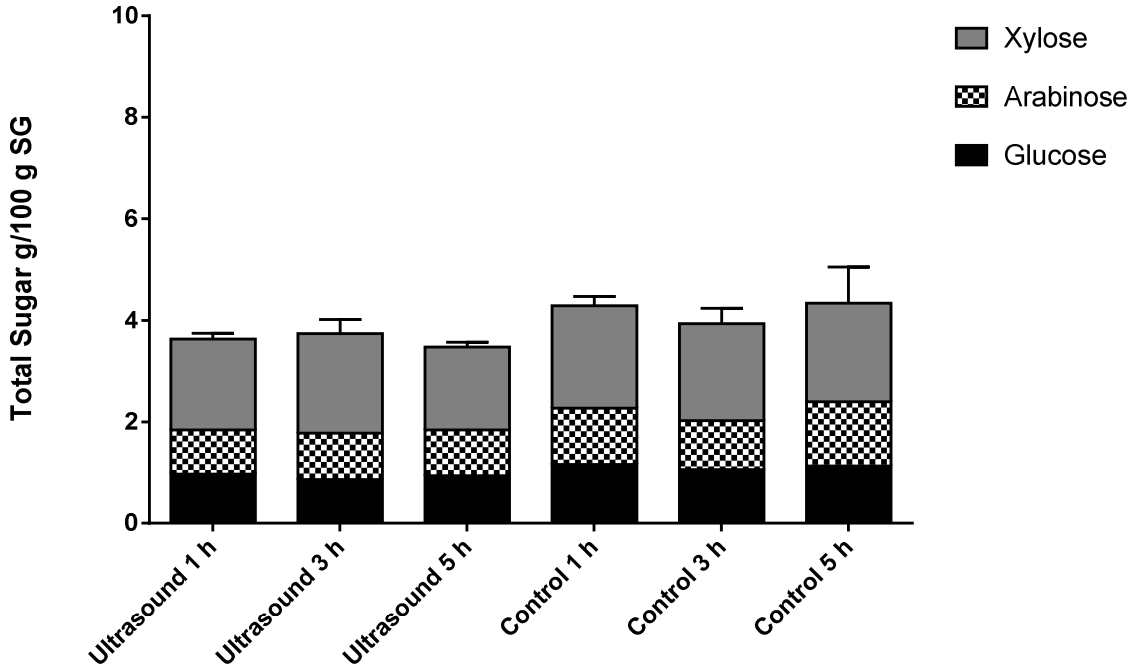


Fig. 5.8 Effects of residence time upon sugar release during enzymolysis of BSG pre-treated with 20kHz ultrasound (Results are means of triplicate treatments with error bars showing SD for total sugar)

Taking into account data from both ultrasonic power and duration of ultrasonic treatment experimentation, it is clear that ultrasound, with the parameters used, is not a viable pre-treatment technique for either BSG or DSG. This in turn suggests that ultrasound, at the applied parameters, is ineffective in the pre-treatment of either malted barley or maize based SG. The inability of ultrasound to pre-treat SG was apparent regardless of increasing the residence time.

As detailed previously, the theory behind ultrasound being able to pre-treat lignocellulose centres upon two specific mechanisms by which ultrasound acts upon the biomass (Yu *et al.*, 2008; Sulman *et al.*, 2011). Firstly, the physical effects of ultrasound lead to in an increase in the porosity

of the lignocellulose matrix through the actions of cavitation bubble implosion upon the surface of the biomass. The increased porosity allows the penetration of cellulolytic enzymes and subsequent enzymolysis. Secondly, the action of ultrasound within an aqueous medium generates hydroxyl radicals though the dissociation of water. The proposed mechanisms of action in combination with observed data tends to suggest that ultrasound was ineffective in SG pre-treatment due to a combination of two potential factors: 1) the applied parameters of frequency and power were not successful in the generation of sufficiently violent cavitation bubbles to physically affect the biomass; and 2) the lack of sufficiently energetic cavitation resulted in production of hydroxyl radicals in quantities insufficient to affect lignin degradation.

Whilst not conducted during this study, there are a number of analytical procedures that could be used to confirm each of the possible factors involved in ultrasound being ineffective in the pre-treatment of SG. With regards to the physical effects of ultrasonic cavitation upon SG, samples pre-treated with ultrasound alongside the respective non-sonicated controls, could be imaged utilising scanning electron microscopy (SEM) which has been shown to be effective in imaging lignocellulose fibres (Behera, Arora and Sharma, 1996). This technique could be used to assess whether or not the application of ultrasound to SG had any physical effects upon the biomass rather than the chemical effects tested for in this study.

The results reported here are in disagreement with previously published results which indicate that ultrasound is effective in the pre-treatment of lignocellulose (Sulman *et al*., 2011; Yu *et al*., 2008). This is likely

to be attributable to the fact that these studies used different ultrasonic frequencies (30 kHz - Sulman *et al*., 2011, 40 kHz - Yu *et al*., 2008) and different sources of lignocellulose (Sunflower husk - Sulman *et al*., 2011, rice hull - Yu *et al*., 2008) than those utilised in this study. Differing ultrasonic frequencies and the biomass type exposed to ultrasound are likely to have a role in how effective ultrasound is in the pre-treatment in lignocellulose.

The level of hydroxyl radical production by the action of ultrasound upon aqueous systems has been shown to increase with frequency (Mason *et al*., 1994; Milne, Stewart and Bremner, 2012). As such it would appear likely that the experimental methodology employed by both Sulman *et al*., (2011) and Yu *et al*., (2008), where ultrasound was applied to lignocellulose at frequencies of 30 kHz and 40 kHz respectively, resulted in enhanced hydroxyl radical formation compared to the ultrasonic frequency reported here (20 kHz).

In order to test the possibility that the ultrasonic setup utilised in this study generates insufficient levels of hydroxyl radicals to affect lignin degradation, the experimental methodology could be expanded to include ultrasonic frequencies that have previously been reported as successful in the pre-treatment of lignocellulose. If increasing ultrasonic frequency led to effective SG pre-treatment quantification of hydroxyl radical production across ultrasonic frequencies of 20, 30 and 40 kHz would give an insight into whether or not 20 kHz ultrasonic pre-treatment was ineffective due to insufficient hydroxyl radical production. Quantification of hydroxyl radicals is possible utilising salicyclic acid dosimetry (Milne, Stewart and Bremner, 2012). The procedure centres upon the fact that the reaction of salicyclic acid

with hydroxyl radicals forms three main products – 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid and catechol. Quantification of the products formed by HPLC can be used to quantify levels of hydroxyl radicals.

In addition to ultrasonic frequency, lignin contents of various types of biomass are likely to play a role in how effective a specific pre-treatment technique is likely to be. The structure of the lignocelluloses structural matrix, where lignin encases the cellulose and hemicellulose component of the micro-fibril, dictates that lignin must be degraded prior to the polysaccharide being accessed and further hydrolysed. As such it follows that higher lignin levels will render biomass more resistant to pre-treatment due to the fact there are simply larger relative amounts of lignin to remove. Sulman *et al*., (2011) exposed sunflower husk to ultrasonic irradiation whilst Yu *et al*. (2008) used rice hull. Both of these sources of biomass have been shown to have similar levels of lignin (Sunflower husk ~18% and Rice hull ~22% - Demirbas, 2008) to the lignin content of SG (~20% as detailed in chapter 3). As such it would appear likely that the ineffective use of ultrasound in the pre-treatment of SG reported here is the result of reduced hydroxyl radical production due to the lower frequency ultrasound used in this study compared to frequencies used by Sulman *et al*., (2011) and Yu *et al*. (2008).

5.3.2 Dilute acid pre-treatment in combination with ultrasound

As has been shown in the earlier part of this chapter, exposing SG to ultrasound alone does not appear to be a viable pre-treatment technique. Therefore, further research sought to assess the applicability of a combined ultrasonic/chemical pre-treatment. The first combined pre-treatment that was

assessed was the use of ultrasonic irradiation in combination with HNO_3. This was achieved by applying the optimal acid concentration (chapter 4) and exposing to ultrasound at 120W at a variety of residence times. In theory, a low temperature acidic pre-treatment conducted at the 50 ℃ used during sonication should lead to a lower production of fermentation inhibitors that are produced during sugar heat degradation.

Total hydrolysate sugars were similar for DSG pre-treated with ultrasound and HNO_3 and that which was treated with only HNO_3 (Fig 5.9). This also extended to a similar level of the component sugars within the hydrolysate (Table 5.9). BSG pre-treated with ultrasound and HNO_3 also showed similar levels of total hydrolysate sugars to BSG treated with solely HNO_3 (Fig.5.10) and again there was no significant difference in the levels of glucose, arabinose and xylose (Table 5.10).

The data indicates that, irrespective of residence time within the reactor, ultrasound does not enhance acidic pre-treatment. Whilst there was an increase in hydrolysate sugars with increasing residence time, this was observed in both samples pre-treated with ultrasound and HNO_3 and those treated with solely HNO_3. As such this can be attributed to increasing SG/ HNO_3 contact time, rather than any action of the applied ultrasound.

The sugars released during pre-treatment comprised both hexose and pentose sugars. Glucose levels in the hydrolysates are likely to be the result of residual SG sugars in combination with the low levels expected to be released during hemicellulose hydrolysis. It is unlikely that they are the result of cellulose degradation as the pre-treatment did not appear to fully

hydrolyse hemicellulose, making it unlikely that the lignin fraction was fully degraded.

The presence of pentoses suggests at least partial hemi-cellulose hydrolysis, however as the data between samples exposed to ultrasound and acid is similar to that of samples only exposed to acid, it is likely that these sugars are the result of low temperature acid hydrolysis and are not attributable to treatment with ultrasound. The effectiveness of acid pre-treatment has been shown to increase with temperature up to a certain point (Esteghlalian *et al.*, 1997). Therefore it is noteworthy that pre-treatment with acid at 50℃ yielded lower levels of hydrolysate sugars than was present in hydrolysates pre-treated with acid at high temperature detailed in Chapter 4.

Table 5.9 – Effects of 20 kHz ultrasound upon specific sugar release during acid pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	**Arabinose**	**Xylose**	**Total**
Ultrasound & HNO_3 - 1 h	0.95 (0.018)	0.35 (0.023)	0.76 (0.016)	2.06 (0.036)
Ultrasound & HNO_3 - 3 h	2.23 (0.274)	1.42 (0.187)	1.69 (0.164)	5.34 (0.652)
Ultrasound & HNO_3 - 5 h	2.15 (0.103)	1.51 (0.096)	1.84 (0.106)	5.5 (0.237)
HNO_3 - 1 h	0.86 (0.234)	0.41 (0.197)	0.81 (0.207)	2.08 (0.684)
HNO_3 - 3 h	2.13 (0.074)	1.51 (0.063)	1.86 (0.048)	5.5 (0.149)
HNO_3 - 5 h	2.36 (0.037)	1.45 (0.022)	1.91 (0.045)	5.72 (0.098)

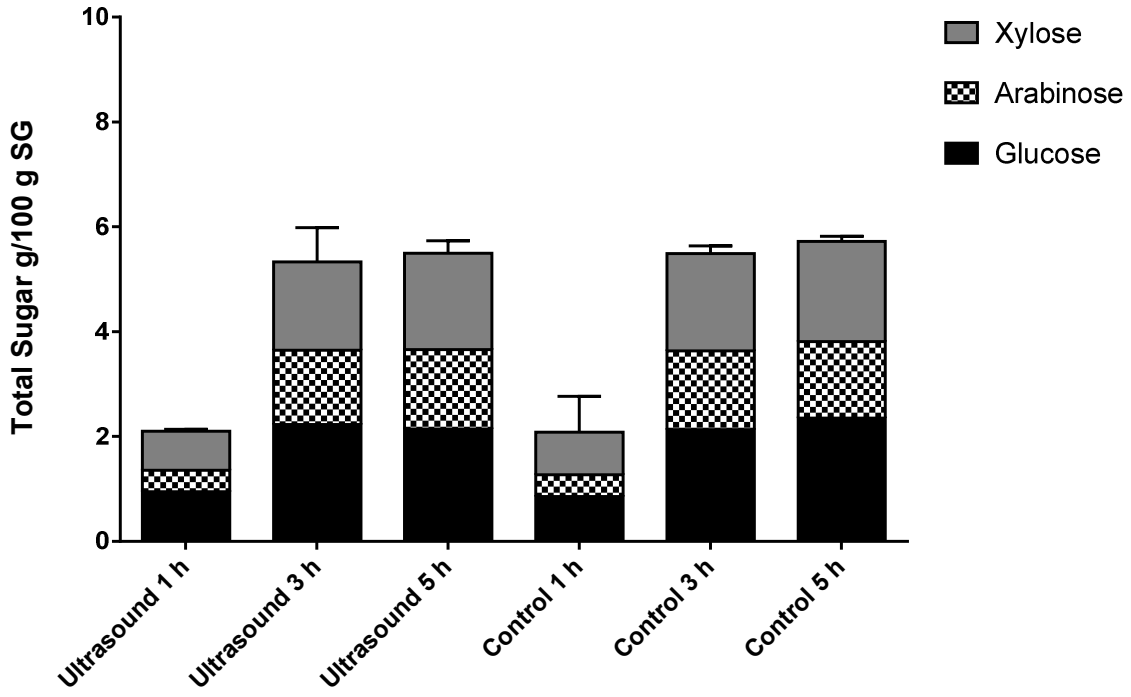


Fig. 5.9 – Effects of 20 kHz ultrasound upon sugar release during acid pre-treatment of DSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.10 – Effects of 20 kHz ultrasound upon specific sugar release during acid pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & HNO_3 - 1 h	1.47 (0.217)	0.41 (0.103)	0.81 (0.038)	2.69 (0.345)
Ultrasound & HNO_3 - 3 h	2.31 (0.238)	0.92 (0.213)	1.35 (0.206)	4.58 (0.654)
Ultrasound & HNO_3 - 5 h	2.99 (0.168)	1.33 (0.142)	1.79 (0.098)	6.11 (0.412)
HNO_3 - 1 h	1.5 (0.601)	0.46 (0.162)	0.76 (0.095)	2.72 (0.894)
HNO_3 - 3 h	2.21 (0.021)	0.89 (0.044)	1.42 (0.068)	4.52 (0.124)
HNO_3 - 5 h	3.05 (0.587)	1.21 (0.271)	1.89 (0.065)	6.15 (0.945)

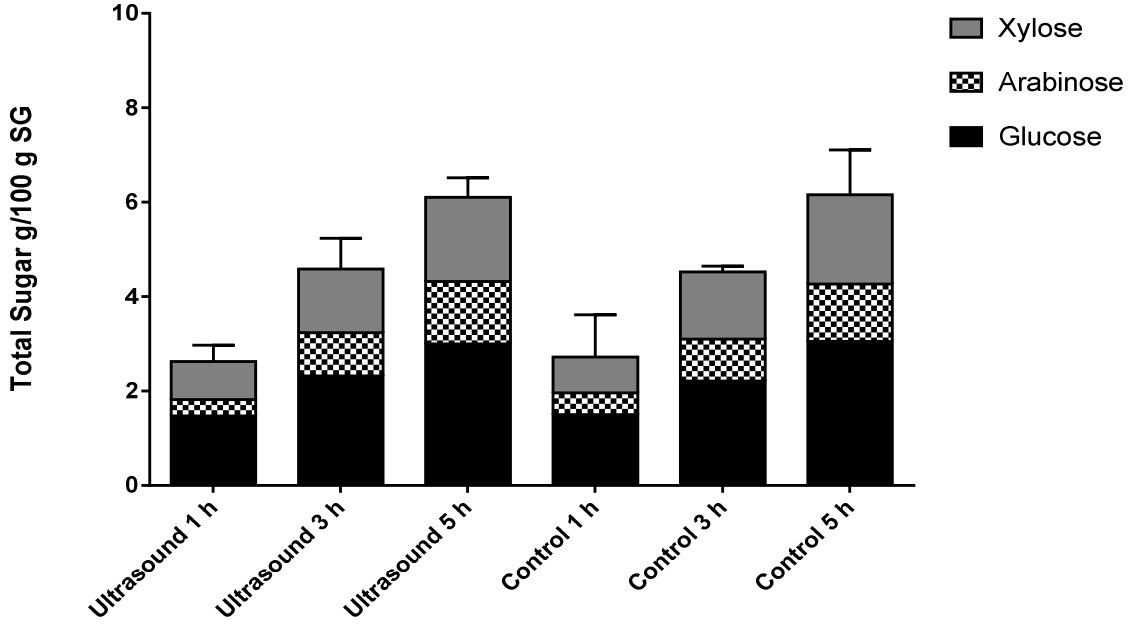


Fig. 5.10 – Effects of 20 kHz ultrasound upon sugar release during acid pre-treatment of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

The effectiveness of a combined ultrasound and acid pre-treatment of SG, was further evaluated by digesting ultrasound treated and control samples with hydrolytic enzymes and the sugars released during enzymolysis were quantified. Enzymatic digestion of DSG that had been pre-treated with ultrasound and HNO_3 showed similar total sugar release during enzymolysis to samples treated with acid only (Fig. 5.11). This also equated to similar levels of glucose, arabinose and xylose between ultrasound treated and non-sonicated controls (Table 5.11). Enzymatic digestion of pre-treated BSG yielded similar results, with total hydrolysate sugar being comparable for both samples pre-treated with ultrasound and HNO_3 and HNO_3 alone (Fig. 5.12). Release of specific sugars was also similar between ultrasound treated BSG and the non-sonicated controls (Table 5.12). Lengthening reactor residence time had no impact on sugars release for either DSG or BSG.

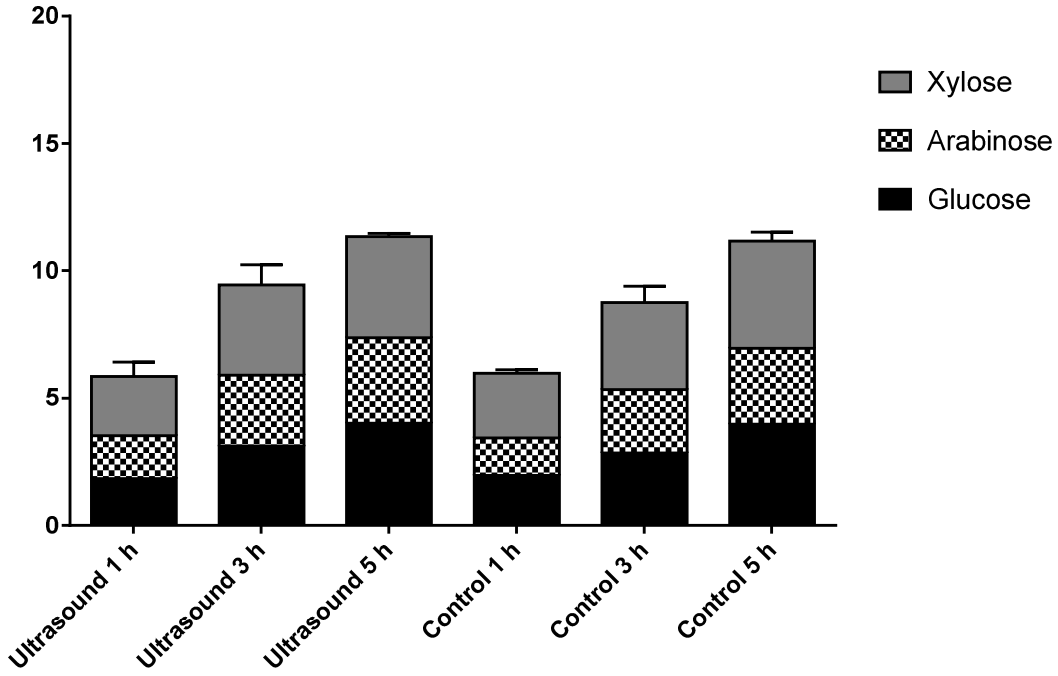


Fig. 5.11 Sugar release during enzymolysis of DSG pre-treated with acid and 20 kHz ultrasound (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.11 Specific sugar release during enzymolysis of DSG pre-treated with acid and 20 kHz ultrasound (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & HNO_3 - 1 h	1.85 (0.198)	1.68 (0.162)	2.32 (0.209)	5.85 (0.562)
Ultrasound & HNO_3 - 3 h	3.11 (0.156)	2.79 (0.237)	3.54 (0.412)	9.44 (0.784)
Ultrasound & HNO_3 - 5 h	4.02 (0.062)	3.34 (0.021)	3.98 (0.088)	11.34 (0.124)
HNO_3 - 1 h	1.96 (0.068)	1.47 (0.078)	2.54 (0.035)	5.97 (0.135)
HNO_3 - 3 h	2.85 (0.098)	2.5 (0.385)	3.41 (0.237)	8.76 (0.641)
HNO_3 - 5 h	3.98 (0.023)	2.96 (0.302)	4.21 (0.056)	11.15 (0.357)

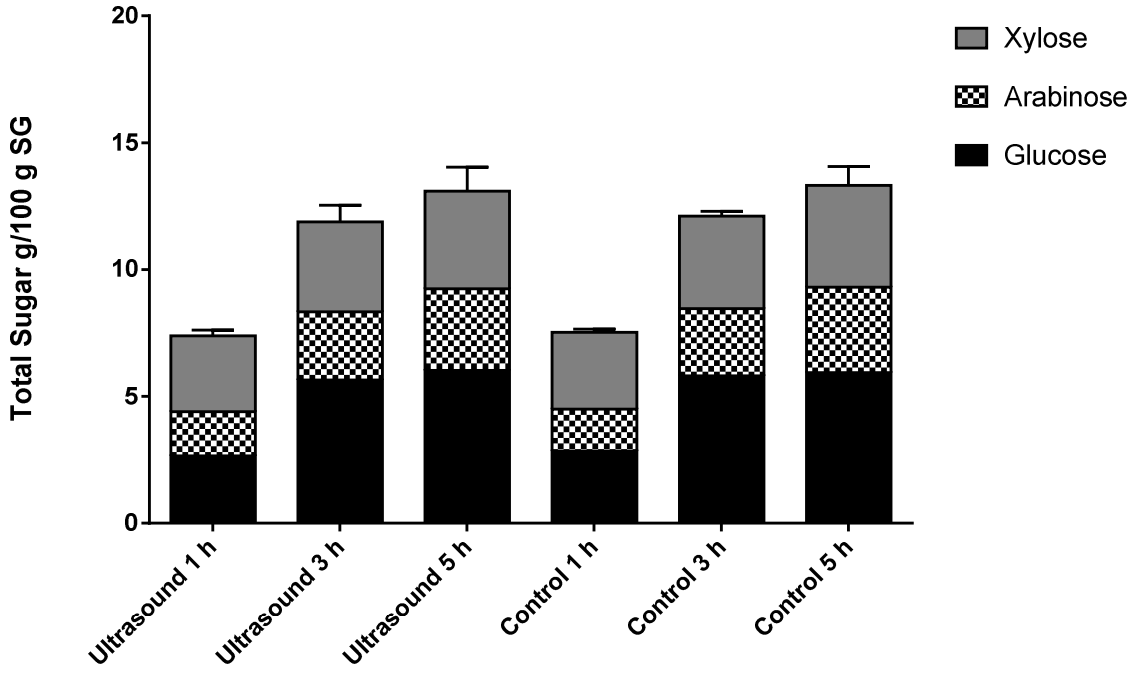


Fig. 5.12 Sugar release during enzymolysis of BSG pre-treated with acid and 20 kHz ultrasound (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.12 Specific sugar release during enzymolysis of BSG pre-treated with acid and 20 kHz ultrasound (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & HNO_3 - 1 h	2.65 (0.106)	1.74 (0.097)	2.98 (0.028)	7.37 (0.235)
Ultrasound & HNO_3 - 3 h	5.64 (0.134)	2.69 (0.197)	3.54 (0.209)	11.87 (0.651)
Ultrasound & HNO_3 - 5 h	6.02 (0.419)	3.21 (0.182)	3.86 (0.278)	13.09 (0.943)
HNO_3 - 1 h	2.84 (0.012)	1.66 (0.048)	3.03 (0.061)	7.53 (0.114)
HNO_3 - 3 h	5.81 (0.127)	2.63 (0.044)	3.66 (0.028)	12.1 (0.189)
HNO_3 - 5 h	5.94 (0.149)	3.37 (0.176)	4.01 (0.481)	13.32 (0.743)

The overall dataset from experimentation seeking to assess the impact of ultrasound upon the efficiency of acid pre-treatment of SG suggests that ultrasound had no effect. This was evident both in the case of sugars released during pre-treatment and those released during subsequent enzymolysis. Whilst sugar hydrolysis was observed following both pre-treatment and enzymolyis the levels released were extremely low. The structural layout of the lignocellulose fibre, with lignin surrounding the polysaccharide component dictates that lignin must be degraded before polysaccharide hydrolysis can occur. As such the increase in sugar yields during enzymolysis indicates at least partial lignin degradation. However as this was observed in both the control and sonicated samples this is clearly as a result of the acid treatment rather than any action of ultrasound.

Increasing the residence time during pre-treatment appeared to result in higher levels of sugars during enzymolysis, however as this was also observed in the control samples, this can be attributed to the actions of the acid rather than ultrasound. The presence of hexose and pentose sugars does suggest partial cellulose and hemi-cellulose hydrolysis, however due to no significant difference between ultrasound treated and control samples, this is likely to be attributable to what is a low temperature acid pre-treatment of the SG rather than the action of ultrasound.

5.3.4 Ultrasound assisted hydrogen peroxide treatment of spent grains

As discussed previously, ultrasound has been shown to increase the effectiveness of alkaline pre-treatment, however there are few reported studies which have sought to assess whether or not ultrasound increases the effectiveness of other widely established chemical pre-treatment techniques,

such as hydrogen peroxide. As a result ultrasound was assessed in terms of its impact upon H_2O_2 pre-treatment of SG.

DSG that was pre-treated with ultrasound and H_2O_2 showed a similar level of total hydrolysate sugars compared to that which was pre-treated with solely H_2O_2 (Fig. 5.13). This corresponded to similar levels of glucose, arabinose and xylose (Table 5.13) release from DSG. Pre-treatment of BSG with ultrasound and H_2O_2 yielded similar results to this observed with DSG, in that total hydrolysate sugars in samples treated with ultrasound and H_2O_2 were no different to samples treated with solely H_2O_2 (Fig. 5.14). This also corresponded to similar levels of glucose, arabinose and xylose (Table 5.14). The level of sugar release for BSG and DSG did not increase with reactor residence time.

Table 5.13 – Effects of 20 kHz ultrasound upon specific sugar release during H_2O_2 pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & H_2O_2 - 1h	0.75 (0.068)	1.02 (0.057)	1.52 (0.098)	3.29 (0.187)
Ultrasound & H_2O_2 - 3h	0.9 (0.017)	1.11 (0.014)	1.57 (0.021)	3.58 (0.069)
Ultrasound & H_2O_2 - 5h	0.85 (0.069)	1.07 (0.071)	1.5 (0.063)	3.41 (0.213)
H_2O_2 - 1 h	0.93 (0.012)	1.01 (0.088)	1.63 (0.101)	3.57 (0.197)
H_2O_2 - 3 h	0.87 (0.052)	1.15 (0.033)	1.58 (0.072)	3.61 (0.161)
H_2O_2 - 5 h	0.99 (0.01)	1.27 (0.032)	1.49 (0.103)	3.76 (0.119)

Table 5.14 – Effects of 20 kHz ultrasound upon specific sugar release during H_2O_2 pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & H_2O_2 - 1 h	0.51 (0.067)	0.97 (0.051)	1.12 (0.113)	2.6 (0.192)
Ultrasound & H_2O_2 - 3 h	0.6 (0.027)	0.81 (0.031)	1.11 (0.048)	2.52 (0.101)
Ultrasound & H_2O_2 - 5 h	0.56 (0.036)	0.8 (0.059)	1.24 (0.134)	2.6 (0.268)
H_2O_2 - 1 h	0.52 (0.096)	0.9 (0.1)	1.01 (0.109)	2.43 (0.302)
H_2O_2 - 3 h	0.58 (0.021)	0.96 (0.095)	1.21 (0.066)	2.75 (0.241)
H_2O_2 - 5 h	0.6 (0.004)	0.84 (0.023)	1.3 (0.018)	2.74 (0.058)

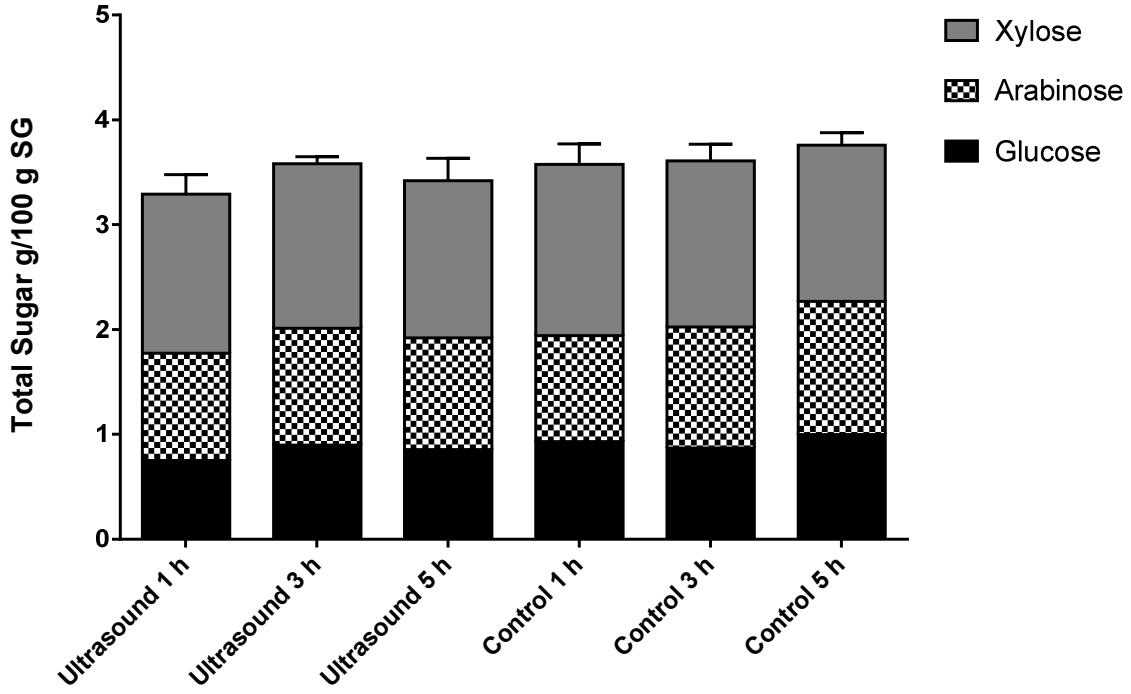


Fig. 5.13 – Effects of 20 kHz ultrasound upon sugar release during H_2O_2 pre-treatment of DSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

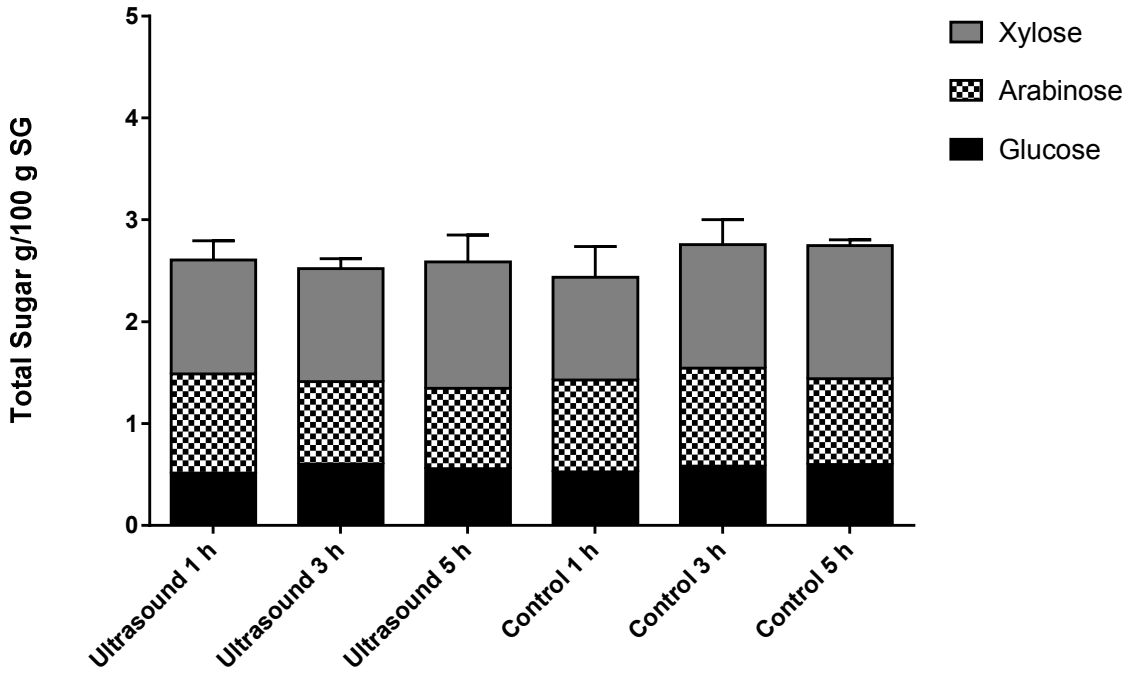


Fig. 5.14 – Effects of 20 kHz ultrasound upon sugar release during H_2O_2 pre-treatment of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Following exposure of DSG and BSG to combined ultrasound and H_2O_2 treatment, there was a limited of pentose sugars. This indicates a degree of hemi-cellulose hydrolysis. However, there was no significant difference in levels of hydrolysate pentose sugars between samples exposed to ultrasound and H_2O_2 and those of the control samples (treated with only H_2O_2). This suggests that ultrasound has little effect upon the release of sugars during pre-treatment with H_2O_2 and that the sugars released were solely attributable to the action of H_2O_2 upon the biomass. As observed in previous ultrasound experiments, there was a low level of glucose release during pre-treatment. However, as appeared to be the case previously, the presence of glucose is likely to be due to residual SG sugars, rather than from cellulose hydrolysis.

In order to further assess pre-treatment efficiency, samples exposed to ultrasound, along with the respective controls, were incubated with cellulolytic enzymes and the hydrolysate sugars were characterised after enzymolysis. During enzymatic digestion of DSG that had been exposed to a combined ultrasonic and H_2O_2 treatment, total hydrolysate sugar levels did rise during enzymolysis, however hydrolysate sugar levels were relatively low (Fig. 5.15). This resulted in relatively low yield of glucose, arabinose and xylose (Table 5.15). This was also observed during enzymatic digestion of BSG that has been pre-treated with a combination of ultrasound and H_2O_2. Whilst there was a degree of sugar release during enzymolysis, the total sugar released was low (Fig. 5.16), alongside low yields of glucose, arabinose and xylose (Table 5.16). The levels of sugars released during enzymatic digestion of DSG and BSG that had been pre-treated with ultrasound and H_2O_2, were similar to those observed in the control sample treated with only H_2O_2.

Table 5.15 Specific sugar release during enzymolysis of DSG pre-treated with 20 kHz ultrasound and H_2O_2 (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & H_2O_2 - 1 h	1.9 (0.218)	1.39 (0.187)	3.01 (0.231)	6.3 (0.674)
Ultrasound & H_2O_2 - 3 h	2.35 (0.1)	1.62 (0.115)	3.57 (0.302)	7.54 (0.511)
Ultrasound & H_2O_2 - 5 h	3.57 (0.318)	2.02 (0.182)	4.24 (0.021)	9.83 (0.497)
H_2O_2 - 1 h	2 (0.051)	1.41 (0.102)	2.99 (0.108)	6.4 (0.274)
H_2O_2 - 3 h	2.51 (0.0.96)	1.72 (0.084)	3.42 (0.135)	7.65 (0.301)
H_2O_2 - 5 h	3.63 (0.367)	2.14 (0.152)	4.31 (0.237)	10.08 (0.671)

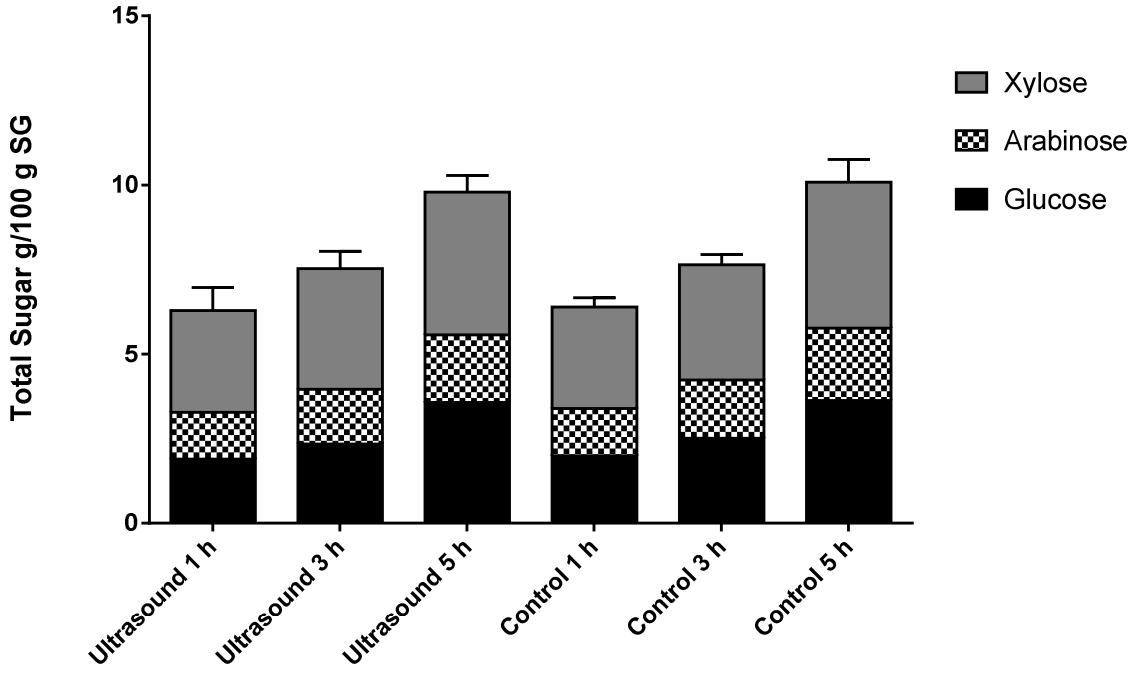


Fig. 5.15 Sugar release during enzymolysis of DSG pre-treated with 20 kHz ultrasound and H_2O_2 (Results are means of triplicate treatments with error bars showing SD for total sugar)

Increasing residence times applied to DSG during pre-treatment, appeared to increase the level of sugars released during enzymolysis, however as this was also observed in the control samples it is likely to be attributable to the increased contact time with hydrogen peroxide rather than exposure to increased ultrasonic reaction time. Increasing residence time appeared to have no impact upon the sugars released during enzymolysis of BSG. The fact that increasing residence time had no effect upon BSG but resulted in increased in sugar release from DSG is likely to be the result of the processing differences between breweries and distilleries. The DSG in this study was from a grain distillery that employs grain cooking prior to

mashing. As discussed previously this is essentially a prior pre-treatment and results in sugars being more easily extractable from DSG than BSG.

During enzymolysis of samples of DSG and BSG that had been treated with ultrasound and H_2O_2, there was a degree of enzymatic glucose release. This suggests that there was some lignin degradation, which rendered the cellulose fraction amenable to enzymatic digestion. However, there was no significant difference between samples exposed to ultrasound and H_2O_2 and the control samples (treated with solely H_2O_2). This indicates that the rendering of the cellulose fraction to enzymatic digestion was the result of treatment with H_2O_2, rather than exposure to ultrasound.

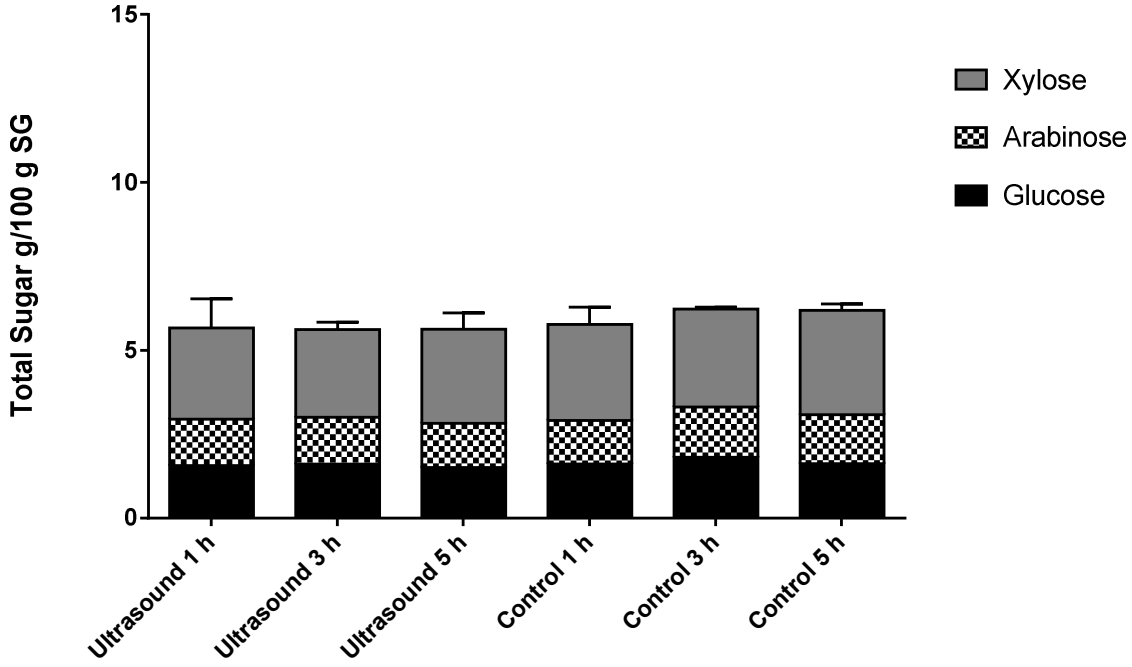


Fig. 5.16 Sugar release during enzymolysis of BSG pre-treated with 20 kHz ultrasound and H_2O_2 (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.16 Specific sugar release during enzymolysis of BSG pre-treated with 20 kHz ultrasound and H_2O_2 (Results are means of triplicate treatments with SD in parenthesis)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & H_2O_2 - 1 h	1.57 (0.401)	1.38 (0.278)	2.72 (0.168)	5.67 (0.874)
Ultrasound & H_2O_2 - 3 h	1.61 (0.071)	1.29 (0.068)	2.86 (0.105)	5.76 (0.214)
Ultrasound & H_2O_2 - 5 h	1.51 (0.158)	1.31 (0.169)	2.8 (0.172)	5.62 (0.488)
H_2O_2 - 1 h	1.61 (0.085)	1.29 (0.098)	2.86 (0.311)	5.76 (0.506)
H_2O_2 - 3 h	1.82 (0.021)	1.5 (0.019)	2.9 (0.016)	6.22 (0.058)
H_2O_2 - 5 h	1.62 (0.011)	1.46 (0.106)	3.1 (0.087)	6.18 (0.197)

Taken as a whole, data from this part of the research project suggests that ultrasound, at this frequency and intensity, has no impact upon the effectiveness of H_2O_2 pre-treatment of SG. There are currently no studies which have reported upon the effect of ultrasound upon hydrogen peroxide pre-treatment of lignocellulose. However ultrasound has been shown to generate hydroxyl radicals through the dissociation of hydrogen peroxide (Huang *et al.*, 1993) (See Eq. 1.9 above). As such the proposed mechanisms by which ultrasound should be able to enhance hydrogen peroxide treatment of lignocellulose are two-fold. Firstly, production of hydroxyl radicals through ultrasonically induced dissociation of water present within the ultrasonic reactor. Secondly, production of hydroxyl radicals through ultrasonically induced dissociation of hydrogen peroxide. Hydroxyl radicals have been shown to degrade lignin though oxidation of C-H bonds contained within subunits (Ek, Gierer and Jansbo, 1989). As such it may be expected that hydroxyl radical production during ultrasonic treatment of SG in the presence

of hydrogen peroxide, would lead to degradation of lignocellulose and the enhancement of hydrogen peroxide pre-treatment of spent grains.

However the results presented here suggest that ultrasound does not increase the effectiveness of hydrogen peroxide pre-treatment of lignocellulose. The data presented here does not lend itself to a definitive reason for this being the case. However it appears likely that ultrasound at the applied parameters was unsuccessful in generating sufficient quantities of hydroxyl radicals (through either dissociation of water or hydrogen peroxide) so as to affect an efficient pre-treatment of SG. This may be attributable to hydrogen peroxide concentrations within the reactor being too low.

There are a number of experimental methodologies that could be employed to test this notion. In the first instance, SG samples could be exposed to ultrasound at 20 kHz and hydrogen peroxide at varying concentrations whilst measuring hydroxyl radical formation. If increasing hydrogen peroxide concentration resulted in effective SG pre-treatment and a correlation was found between increased hydrogen peroxide concentration and hydroxyl radical production, this would give further insight as to the reasons for the ineffectiveness of ultrasound in augmenting hydrogen peroxide pre-treatment of SG reported here.

5.3.5 Ultrasound assisted ozonolysis of spent grains

Another form of chemical pre-treatment, upon which the effects of ultrasound, has not been documented within the primary literature, is treatment with ozone. Ozone is highly oxidising and in theory the combined effects of ultrasonic cavitation and ozone should be more effective in the pre-treatment of SG than pre-treatment with solely ozone. Consequently, ultrasound was evaluated as to its effect upon ozone pre-treatment of SG.

DSG that was exposed to ultrasonic and O_3 treatment showed similar total hydrolysate sugars to samples exposed to solely O_3 (Fig. 5.17). This was also observed in BSG treated with ultrasound and O_3, with hydrolysates showing similar total sugar levels compared to BSG treated with O_3 alone (Fig. 5.18). The sugars released during combined ultrasonic and O_3 treatment comprised solely glucose and did not vary significantly between samples exposed to ultrasound and O_3 and those exposed to solely O_3. This was evident for both BSG (Table 5.17) and DSG (Table 5.18) and as previously discussed is likely to be attributable to residual SG sugars. Glucose release for DSG and BSG did not increase with reactor residence time. The lack of pentose sugars following treatment of SG indicates that treatment with ultrasound and O_3 or O_3 alone, does not result in hemi-cellulose hydrolysis. However, ozone has a specificity towards oxidising double bonds, as such it is likely that ozone will have a tendency to attack lignin rather than hemi-cellulose.

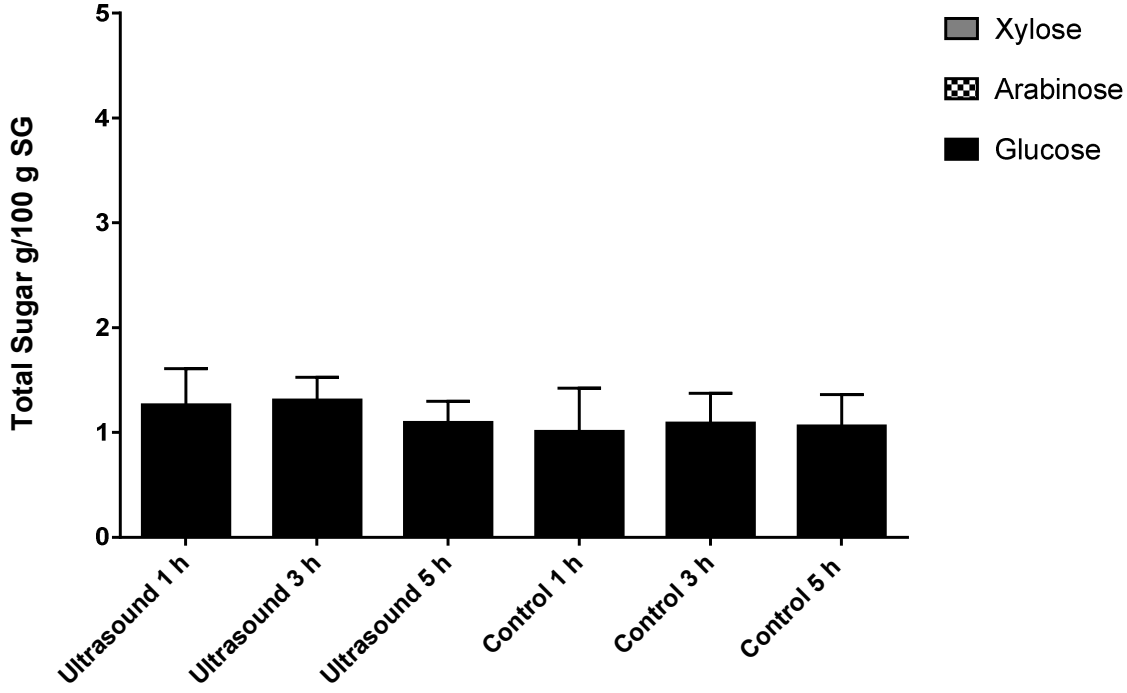


Fig. 5.17 – Effects of 20 kHz ultrasound upon sugar release during O_3 pre-treatment of DSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.17 – Effects of 20 kHz ultrasound upon specific sugar release during O_3 pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis. Glucose concentrations from samples treated with ultrasound are not significantly different to the respective control, independent t-test, significance level $p>0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & O_3 - 1 h	1.27 (0.345)	0 (0)	0 (0)	1.27 (0.345)
Ultrasound & O_3 - 3 h	1.31 (0.214)	0 (0)	0 (0)	1.31 (0.214)
Ultrasound & O_3 - 5 h	1.1 (0.199)	0 (0)	0 (0)	1.1 (0.199)
O_3 - 1 h	1.01 (0.412)	0 (0)	0 (0)	1.01 (0.412)
O_3 - 3 h	1.09 (0.287)	0 (0)	0 (0)	1.09 (0.287)
O_3 - 5 h	1.06 (0.301)	0 (0)	0 (0)	1.06 (0.301)

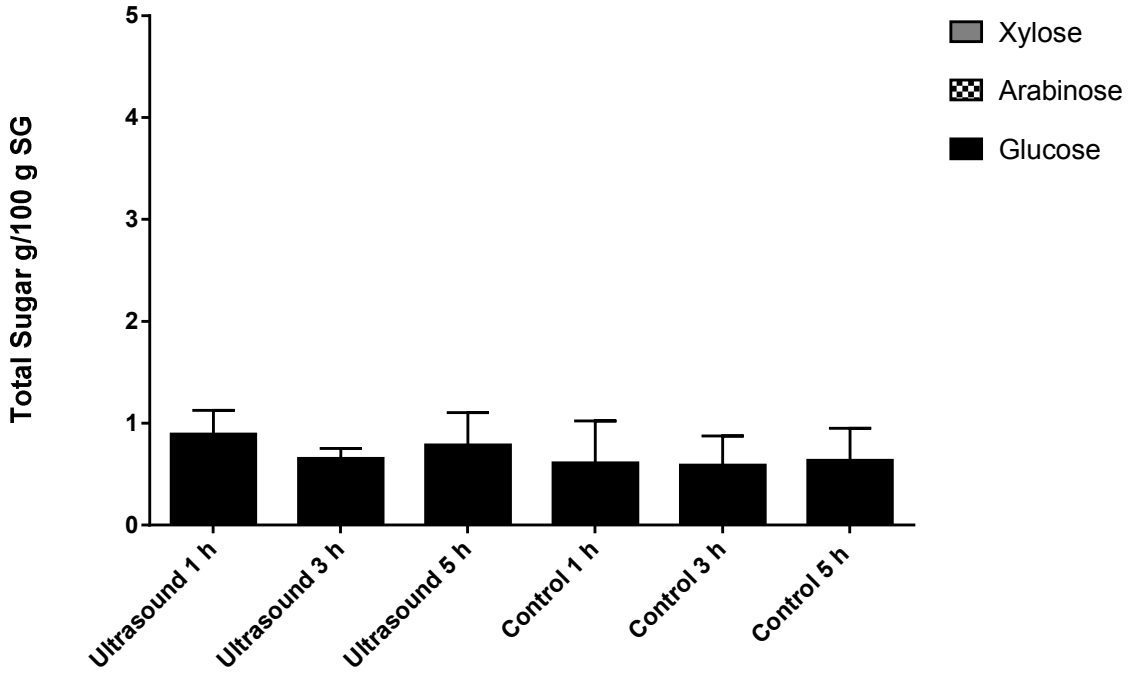


Fig. 5.18 – Effects of 20 kHz ultrasound upon sugar release during O_3 pre-treatment of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.18 – Effects of 20 kHz ultrasound upon specific sugar release during O_3 pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are not significantly different to the respective control, independent t-test, significance level $p>0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & O_3 - 1 h	0.89 (0.231)	0 (0)	0 (0)	0.89 (0.231)
Ultrasound & O_3 - 3 h	0.65 (0.099)	0 (0)	0 (0)	0.65 (0.099)
Ultrasound & O_3 - 5 h	0.79 (0.314)	0 (0)	0 (0)	0.79 (0.314)
O_3 - 1 h	0.61 (0.412)	0 (0)	0 (0)	0.61 (0.412)
O_3 - 3 h	0.59 (0.289)	0 (0)	0 (0)	0.59 (0.289)
O_3 - 5 h	0.64 (0.314)	0 (0)	0 (0)	0.64 (0.314)

Pre-treatment efficiency was further assessed by the enzymatic digestion of samples exposed to ultrasound and O_3 and the respective controls. The level of sugar release during enzymolysis is, in part, governed by the effectiveness of the employed pre-treatment technique. Due to the position of lignin within the lignocelluloses matrix, in that it surrounds cellulose and hemicellulose, it must be removed or at least partially degraded, before either component can be hydrolysed. An effective pre-treatment will fully degrade lignin and allow hydrolysis of the cellulose and hemicelluloses fibres. It follows that quantifying sugar release during enzymolysis gives an indication as to the efficiency of the pre-treatment.

In the case of DSG, total hydrolysate sugar increased significantly during enzymolysis. This was observed in both DSG pre-treated with ultrasound and O_3 and that treated with solely O_3. However, the samples treated with ultrasound showed an increased sugar yield during enzymolysis (Fig. 5.19). This corresponded with an increased yield of glucose, arabinose and xylose in DSG treated with ultrasound and O_3, compared to samples treated with O_3 alone (Table 5.19). These results were similar to those observed during enzymolysis of BSG that had been pre-treated with ultrasound and O_3, with samples showing a higher total sugar concentration compared to BSG pre-treated with solely O_3 (Fig. 5.20). This also translated to an increased yield of glucose, arabinose and xylose (Table 5.20).

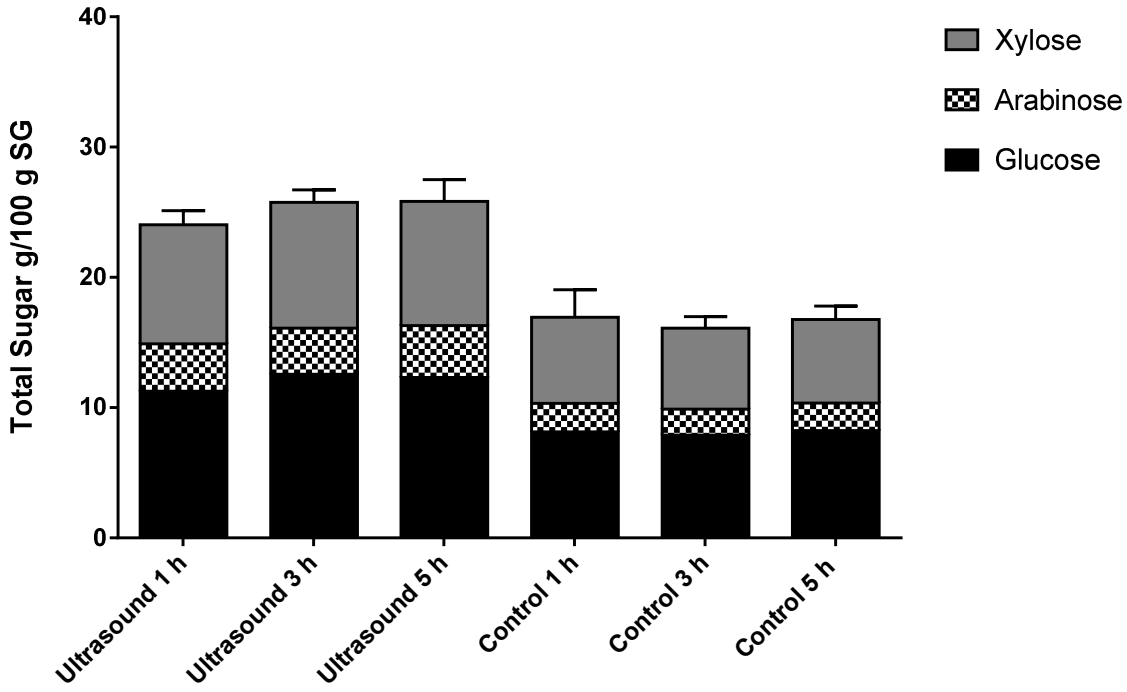


Fig. 5.19 Sugar release during enzymolysis of DSG pre-treated with 20 kHz ultrasound and O_3 (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.19 – Specific sugar release during enzymolysis of DSG pre-treated with 20 kHz ultrasound and O_3 (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are significantly different to the respective control, independent t-test, significance level $p<0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & O_3 - 1 h	11.25 (0.512)	3.65 (0.254)	9.12 (0.206	24.02 (1.09)
Ultrasound & O_3 - 3 h	12.54 (0.638)	3.54 (0.305)	9.68 (0.116)	25.76 (0.945)
Ultrasound & O_3 - 5 h	12.32 (0.566)	3.98 (0.412)	9.54 (0.776)	25.84 (1.650)
O_3 - 1 h	8.14 (0.955)	2.17 (0.362)	6.61 (0.876)	16.92 (2.13)
O_3 - 3 h	7.89 (0.361)	1.99 (0.334)	6.22 (0.109)	16.1 (0.874)
O_3 - 5 h	8.23 (0.502)	2.11 (0.0641)	6.42 (0.597)	16.76 (1.032)

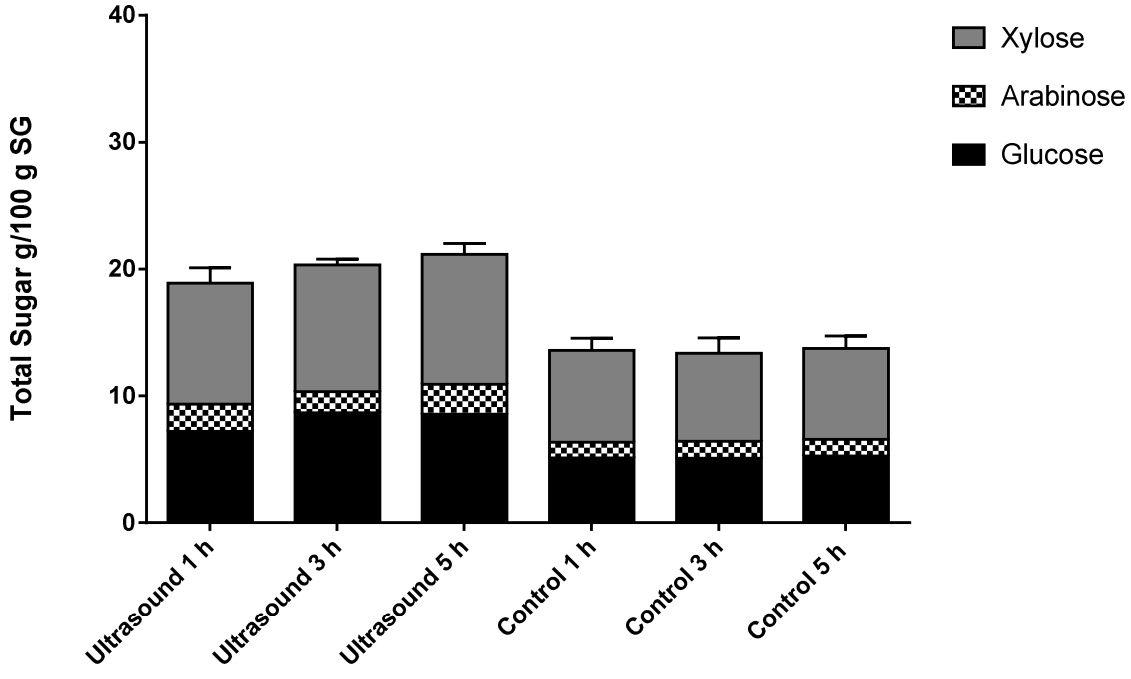


Fig. 5.20 Sugar release during enzymolysis of BSG pre-treated with 20 kHz ultrasound and O_3 (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.20 – Specific sugar release during enzymolysis of BSG pre-treated with 20 kHz ultrasound and O_3 (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are significantly different to the respective control, independent t-test, significance level $p<0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound & O_3 - 1 h	7.21 (0.591)	2.14 (0.312)	9.54 (0.285)	18.89 (1.21)
Ultrasound & O_3 - 3 h	8.65 (0.203)	1.7 (0.095)	9.98 (0.162)	20.33 (0.450)
Ultrasound & O_3 - 5 h	8.55 (0.217)	2.37 (0.295)	10.23 (0.326)	21.15 (0.870)
O_3 - 1 h	5.12 (0.452)	1.24 (0.106)	7.24 (0.541)	13.6 (0.940)
O_3 - 3 h	5.09 (0.234)	1.32 (0.234)	6.97 (0.681)	13.38 (1.179)
O_3 - 5 h	5.28 (0.27)	1.3 (0.182)	7.17 (0.635)	13.75 (0.982)

The overall data set for pre-treatment of SG with ultrasound and ozone indicates that ultrasound significantly enhances ozonolysis of SG. Samples which were pre-treated with solely ozone showed extremely low levels of hydrolysate sugars comprising only glucose. However this would be expected as ozone has a high specificity towards reacting with compounds which contain high levels of double bonds (e.g. C=C, C=N, N=N) (García-Cubero *et al*., 2009). As such its main mechanism of action would be to oxidise and remove lignin from lignocellulose structural matrix. This notion was borne out following enzymolysis with control samples showing a relatively high release of sugars following enzymatic digestion. This would only be expected to occur following at least partial lignin degradation.

During pre-treatment with ultrasound and O_3, there was limited release of sugars, which suggests that the treatment did not hydrolyse cellulose or hemi-cellulose. As was the case with control samples this may

be expected as ozone has specificity towards lignin with its high degree of double bonds. This is confirmed by sugar characterisation following enzymolysis which shows that there was a significant degree of lignin degradation due to high release of sugars. Whilst this was observed in samples treated with ultrasound and O_3 and those treated with solely O_3, the increased level of sugars released during enzymatic digestion of samples treated with ultrasound and O_3 compared to those treated with solely O_3, suggests that ultrasound significantly enhances lignin degradation during ozone pre-treatment of SG. This subsequently results in an increased sugar yield during enzymolysis. The magnitude of this enhancement was not increased by longer reactor residence time, with no significant difference between SG treated for 1, 3 and 5 h. Although not conducted during this study, the apparent increase of lignin degradation caused by the ultrasound during ozonolysis of SG could be further characterised through quantification of lignin degradation products (e.g. vanillin, coumaryl and sinapyl alcohol) by HPLC as described in Chapter 4.

Whilst the data presented does not lend itself to a definitive conclusion as to why ultrasound enhances ozonolysis of SG, it is likely that this can be attributed to enhanced hydroxyl radical formation within the reactor. Pre-treatment with ultrasound and ozone can be thought of as being able to generate hydroxyl radicals through two mechanisms - through the ultrasonic dissociation of water within the reactor and ultrasonic dissociation of ozone (see Eq. 1.10 above) (Gogate and Pandit, 2004). As such it would appear likely that the enhanced effectiveness of pre-treatment with

ultrasound and ozone compared to solely ozone is likely to be due to the actions of hydrolxyl radicals and associated degradation of lignin.

5.3.6 Ozonolysis of spent grains in combination with ultrasound and hydrogen peroxide

As has been detailed earlier in this chapter, combined ozone and ultrasound pre-treatment improves the enzymatic digestion of both BSG and DSG, compared to enzymatic digestion of SG pre-treated with only ozone. However, the use of ultrasound in combination with ozone is not as effective as the conventional pre-treatment techniques detailed in chapter 4. Thus further work sought to enhance the pre-treatment of SG, through the evaluation of the use of ultrasound, ozone and hydrogen peroxide in a combined technique. This was achieved by applying ultrasonic irradiation to the SG suspended in a dilute solution of H_2O_2, whilst diffusing O_3 into the ultrasonic reactor.

Results from this particular piece of research showed a marked improvement in the efficiency of ultrasonic pre-treatment. This was evident with DSG and BSG. The addition of H_2O_2 and O_3 into the reactor caused a marked increase in the sugars released during pre-treatment and this also carried over to a further enhancement of the sugars released during enzymolysis. This suggests that the addition of H_2O_2 and O_3 increased the level of lignin degradation compared to previously detailed ultrasound research.

DSG that was exposed to ultrasound (20kHz and 120W), O_3 and H_2O_2 pre-treatment, showed an increase in the total sugar release compared to

pre-treatment with O_3 and H_2O_2 (Fig. 5.21). This translated to an increased yield of arabinose and xylose (Table 5.21) and was evident for all of the reactor residence times that were studied (1,3 and 5h). However, increasing reactor residence time above 1h did not improve sugar yield. This was similar to combined ultrasonic, O_3 and H_2O_2 pre-treatment of BSG, which also showed an increase in total sugar release during pre-treatment compared to samples treated with O_3 and H_2O_2 (Fig. 5.22) and an increased yield of arabinose and xylose (Table 5.22). The levels of arabinose and xylose release during treatment with O_3 and H_2O_2, indicates a level of hemi-cellulose hydrolysis, however the increased yield of pentose sugars released during pre-treatment with ultrasound, O_3 and H_2O_2 suggests that ultrasound improved hemi-cellulose hydrolysis.

However, treatment with ultrasound, O_3 and H_2O_2 yielded similar levels of glucose compared to treatment with O_3 and H_2O_2. This suggests that both treatment regimes did not hydrolyse significant amounts of cellulose and indicates that ultrasound improves hemi-cellulose hydrolysis but not cellulose hydrolysis. This is likely to be due to the fact that whilst treatment with ultrasound increased hemicellulose hydrolysis it did not hydrolyse all of hemicellulose fraction. This results in residual hemicellulose surrounding the cellulose fibre and protecting it from hydrolysis.

Table 5.21 – Effects of 20 kHz ultrasound upon specific sugar release during combined H_2O_2/O_3 pre-treatment of DSG (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are significantly different to the respective control, independent t-test, significance level $p<0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound, O_3 & H_2O_2 – 1h	1.32 (0.236)	2.65 (0.389)	6.79 (0.489)	10.76 (1.21)
Ultrasound, O_3 & H_2O_2 – 3h	1.12 (0.12)	2.03 (0.268)	6.97 (0.322)	10.12 (0.89)
Ultrasound, O_3 & H_2O_2 – 5h	1.41 (0.106)	2.51 (0.485)	7.01 (0.513)	10.93 (1.32)
O_3 and H_2O_2 – 1h	0.79 (0.144)	1.03 (0.162)	4.23 (0.874)	6.05 (1.13)
O_3 and H_2O_2 – 3h	0.92 (0.068)	1.12 (0.217)	4.13 (0.757)	6.17 (1.06)
O_3 and H_2O_2 – 5h	0.87 (0.097)	1.32 (0.114)	4.47 (0.589)	6.66 (0.73)

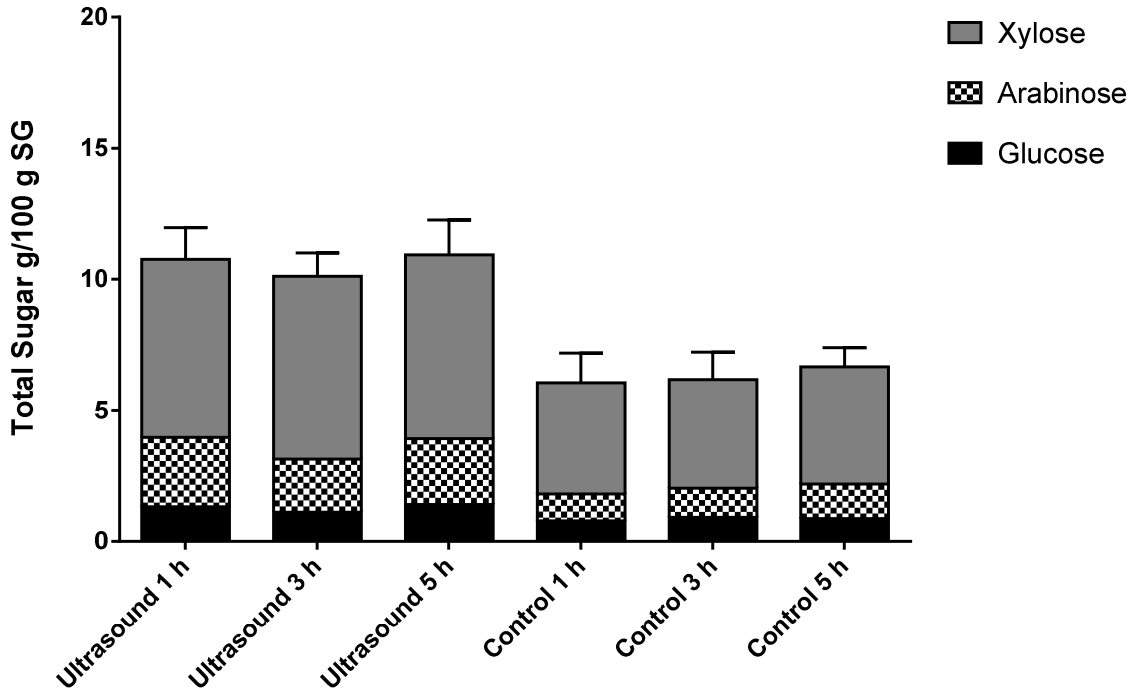


Fig. 5.21 Effects of 20 kHz ultrasound upon sugar release during combined H_2O_2/O_3 pre-treatment of DSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.22 Effects of 20 kHz ultrasound upon specific sugar release during combined H_2O_2/O_3 pre-treatment of BSG (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are significantly different to the respective control, independent t-test, significance level $p<0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound, O_3 & H_2O_2 – 1h	1.33 (0.261)	1.76 (0.068)	4.33 (0.471)	7.41 (0.874)
Ultrasound, O_3 & H_2O_2 – 3h	1.11 (0.188)	1.98 (0.361)	4.65 (0.512)	7.75 (1.054)
Ultrasound, O_3 & H_2O_2 – 5h	1.27 (0.156)	1.58 (0.187)	4.76 (0.103)	7.6 (0.421)
O_3 and H_2O_2 – 1h	0.87 (0.089)	1.03 (0.166)	2.94 (0.381)	4.85 (0.641)
O_3 and H_2O_2 – 3h	0.93 (0.314)	1.11 (0.231)	2.66 (0.624)	4.7 (1.167)
O_3 and H_2O_2 – 5h	0.96 (0.106)	1.09 (0.162)	2.71 (0.268)	4.76 (0.565)

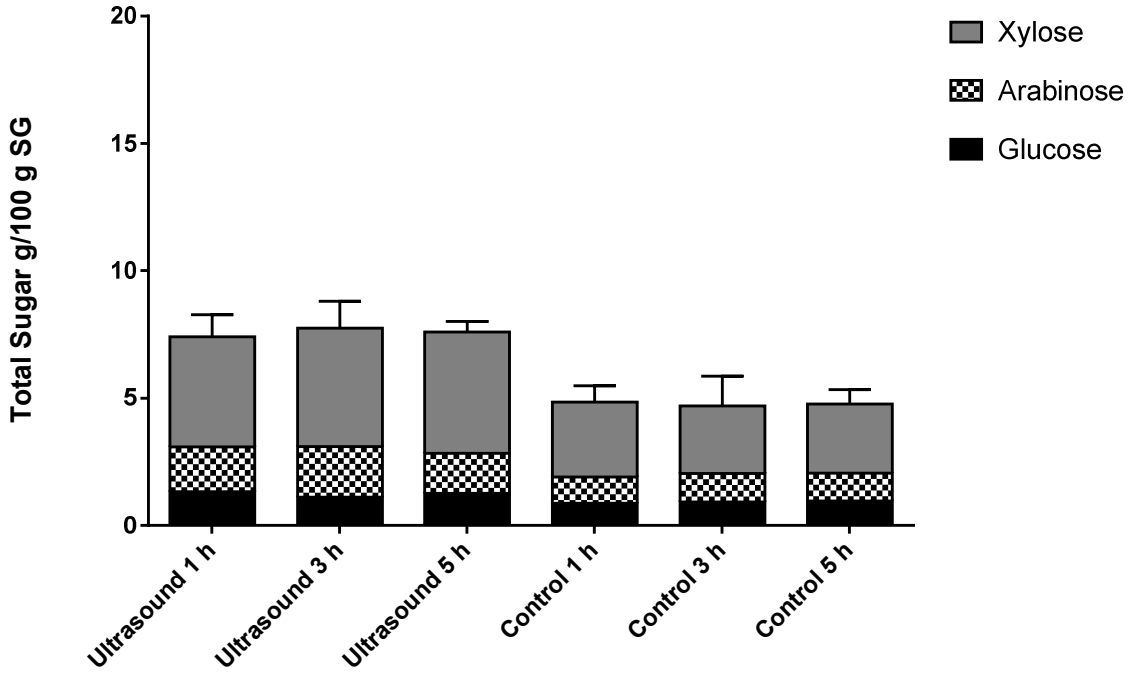


Fig. 5.22 – Effects of 20 kHz ultrasound upon sugar release during combined H_2O_2/O_3 pre-treatment of BSG (Results are means of triplicate treatments with error bars showing SD for total sugar)

Pre-treatment efficiency was further assessed by subjecting samples exposed to ultrasound O_3 and H_2O_2, along with their respective controls, to further hydrolysis enzymatically. Following enzymolysis of DSG, samples that had been pre-treated with ultrasound, O_3 and H_2O_2 showed an increase total sugar yield compared to those treated with only O_3 and H_2O_2 (Fig. 5.23). This was also observed with BSG samples pre-treated with ultrasound, O_3 and H_2O_2 showing an increased level of total hydrolysate sugars during enzymolysis compared to samples treated with O_3 and H_2O_2 (Fig. 5.24). In the case of both DSG (Table 5.23) and BSG (Table 5.24), there was an increased yield of all three of the main hydrolysate sugars (glucose, arabinose and xylose) when SG was pre-treated using ultrasound, O_3 and

H_2O_2 compared to just O_3 and H_2O_2. The data suggests that whilst limited cellulose hydrolysis was observed during the pre-treatment stage, treatment with O_3 and H_2O_2 was effective in degrading lignin and increasing the susceptibility of cellulose to enzymatic hydrolysis. The combined pre-treatment using ultrasound, O_3 and H_2O_2 was more effective in this process than using O_3 and H_2O_2 in the absence of ultrasound. This indicates that ultrasound enhances combined ozone and hydrogen peroxide pre-treatment of SG. This subsequently results in an increased sugar yield during pre-treatment and enzymolysis. The magnitude of this enhancement was not increased by lengthening reactor residence time, with little difference between SG treated for 1, 3 and 5 h.

Table 5.23 Specific sugar release during enzymolysis of DSG pre-treated with 20 kHz ultrasound, H_2O_2 and O_3 (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are significantly different to the respective control, independent t-test, significance level $p<0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound, O_3 & H_2O_2 – 1h	17.21 (0.562)	4.2 (0.789)	12.84 (0.384)	34.25 (1.56)
Ultrasound, O_3 & H_2O_2 – 3h	17.35 (0.441)	4.94 (0.682)	12.01 (0.955)	34.3 (2.01)
Ultrasound, O_3 & H_2O_2 – 5h	16.87 (1.012)	3.81 (0.632)	11.94 (0.305)	32.62 (1.89)
O_3 and H_2O_2 – 1h	10.64 (0.522)	2.79 (0.432)	7.59 (0.238)	21.02 (1.03)
O_3 and H_2O_2 – 3h	11.87 (0.983)	2.36 (0.561)	6.99 (0.268)	21.22 (1.358)
O_3 and H_2O_2 – 5h	11.34 (0.185)	2.58 (0.481)	7.21 (0.335)	21.13 (0.993)

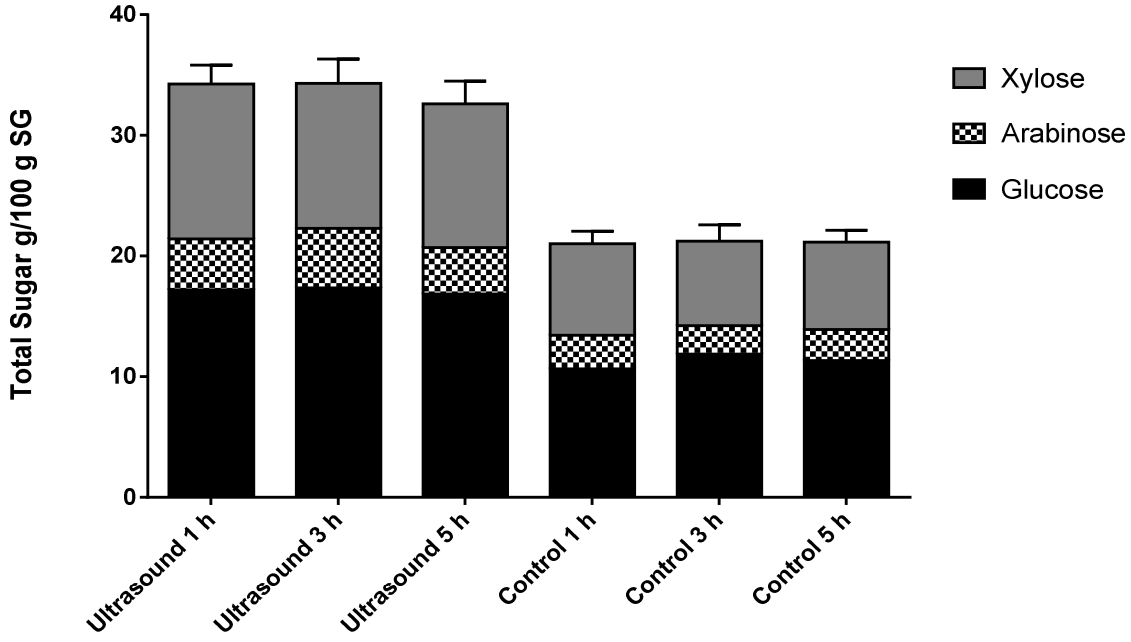


Fig. 5.23 Sugar release during enzymolysis of DSG pre-treated with 20 kHz ultrasound, H_2O_2 and O_3 (Results are means of triplicate treatments with error bars showing SD for total sugar)

Table 5.24 Specific sugar release during enzymolysis of BSG pre-treated with 20 kHz ultrasound, H_2O_2 and O_3 (Results are means of triplicate treatments with SD in parenthesis. Concentrations of each of the hydrolysate sugars from samples treated with ultrasound are significantly different to the respective control, independent t-test, significance level $p<0.05$)

Treatment	Hydrolysate sugar concentration (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
Ultrasound, O_3 & H_2O_2 – 1h	13.98 (1.213)	4.56 (0.662)	8.56 (0.208)	27.1 (2.11)
Ultrasound, O_3 & H_2O_2 – 3h	12.14 (0.523)	4.32 (0.801)	8.98 (0.238)	25.44 (1.451)
Ultrasound, O_3 & H_2O_2 – 5h	13.01 (1.023)	4.89 (0.284)	9.32 (0.501)	27.22 (1.64)
O_3 and H_2O_2 – 1h	8.12 (0.185)	3.01 (0.411)	5.35 (0.387)	16.48 (0.978)
O_3 and H_2O_2 – 3h	8.66 (0.741)	2.89 (0.324)	5.01 (0.255)	16.56 (1.158)
O_3 and H_2O_2 – 5h	7.98 (0.175)	2.91 (0.274)	5.12 (0.410)	16.01 (0.874)

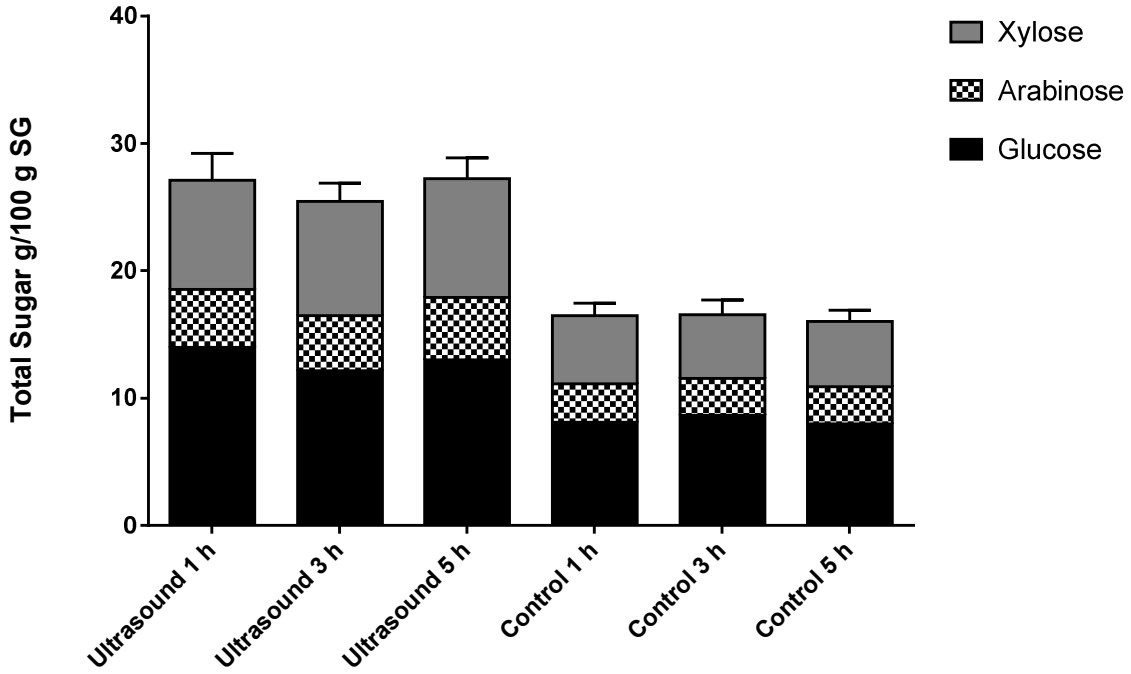


Fig. 5.24 Sugar release during enzymolysis of BSG pre-treated with 20 kHz ultrasound, H_2O_2 and O_3 (Results are means of triplicate treatments with error bars showing SD for total sugar)

Both ozone and hydrogen peroxide are, by their nature, highly oxidising. The addition of hydrogen peroxide to ozone treatment of lignocellulose appears to significantly enhance the effectiveness of the pre-treatment. This is evidenced by the fact that treatment with ozone and hydrogen peroxide conducted as the control sample in this set of experiments was significantly more effective in pre-treating SG than the control sample utilised during earlier research using solely ozone. Coupled with this, ultrasound enhances both ozone pre-treatment of SG, as well as combined ozone/ hydrogen peroxide treatment of SG. It is conceivable that

ultrasound augmented the production of highly reactive oxygen species that contributed to SG lignocellulose degradation.

As has been discussed previously in this chapter, the use of ozone in combination with hydrogen peroxide has received little attention within the primary literature with regards to the pre-treatment of lignocellulose. It is however one of a number of AOP's that are established techniques in the field of industrial effluent water treatment (Vogelpohl and Kim, 2004). The reaction between hydrogen peroxide and ozone ultimately results in the production of hydroxyl radicals through the consumption of ozone and hydrogen peroxide (Forni *et al*., 1982; Sehested *et* al., 1982; Buhler *et al*., 1984).

Control samples that were exposed to pre-treatment with ozone and hydrogen peroxide showed a relatively low level of sugar release following pre-treatment and enzymolysis. However, the levels of pentose sugar release following pre-treatment was high enough to suggest a degree of hemicellulose hydrolysis. This in turn suggests a degree of lignin degradation as the structural arrangement of hemicellulose within the micro-fibril dictates that lignin must be removed to render hemicellulose amenable to hydrolysis. Coupled with this the level of glucose release during enzymolysis of the control samples pre-treated with ozone and hydrogen peroxide suggests a degree of cellulose hydrolysis. This provides additional evidence that combined ozone and hydrogen peroxide results in relatively effective lignin degradation. Cellulose occupies the central region within the lignocellulose micro-fibril and as such it must be accessible in order for it to be hydrolysed. Within control samples lignin degradation appears likely to have occurred

through a combination of hydroxyl radical attack (generated through the reaction of ozone and hydrogen peroxide) combined with the oxidative characteristic of each compound.

Pre-treatment of SG with ultrasound in combination with ozone and hydrogen peroxide showed an enhanced level of hydrolysate sugars following both pre-treatment and enzymolysis. This in turn suggests that the ultrasonic treatment employed led to enhanced lignin degradation as lignin degradation is a pre-requisite to sugar extraction. Additionally, samples pre-treated with ultrasound showed higher levels of pentose sugars indicating an enhanced level of hemicellulose hydrolysis. However levels of pentose sugars were observed to be lower than the values reported in Chapter 4 suggesting a level of unhydrolysed hemicellulose. Similar glucose levels observed between sonicated and control samples following pre-treatment suggest that ultrasound did not increase cellulose hydrolysis. This is likely to be attributable to the fact there was residual hemicellulose covering the exterior of the cellulose fibres and protecting it from hydrolysis.

As was the case in previous experiments SG pre-treatment with ultrasound, ozone and hydrogen peroxide was more effective in extracting sugar from DSG than BSG. This is likely to again relate to processing difference between breweries and distilleries as previously discussed in Chapter 3. The DSG used in this study was from a grain distillery that employs cereal cooking prior to mashing. This in effect exposes the grains to a prior pre-treatment step and renders the carbohydrate component of this particular DSG more amenable to hydrolysis.

Ultrasound has been shown to generate hydroxyl radicals through dissociation of either ozone or hydrogen peroxide (Huang et al., 1993; Gogate and Pandit, 2004). Although it has not been proven conclusively within the field of sono-chemistry that ultrasound can enhance hydroxyl radical production during the reaction between ozone and hydrogen peroxide, it would appear likely that it provides a more direct route to hydroxyl radical production than the previously discussed series of reactions between ozone and hydrogen peroxide in the absence of ultrasound.

There are a number of possible avenues for increased production of hydroxyl radicals during sonication of SG in the presence of ozone and hydrogen peroxide. As discussed previously ultrasound can generate hydroxyl radicals through dissociation of ozone and hydrogen peroxide. Additionally, water present within the reactor can also dissociate which provides another route to hydroxyl radical production. These factors coupled with the fact that hydroxyl radicals have been shown to degrade lignin (Ek, Gierer and Jansbo, 1989) suggest that the increased effectiveness of pre-treatment of SG with ultrasound, ozone and hydrogen peroxide compared to pre-treatment with ozone and hydrogen peroxide is likely to be attributable to increased hydroxyl radical production though ultrasonically induced dissociation of ozone and hydrogen peroxide.

However, further insight could be gained by repeating the experimental procedure reported here and quantifying hydroxyl radical production as described previously. If there was a greater level of hydroxyl radical production using ultrasound, ozone and hydrogen peroxide during pre-treatment than that observed using only ozone and hydrogen peroxide,

this would go some way to proving that ultrasound enhances combined ozone and hydrogen peroxide pre-treatment of SG due to increasing the production of hydroxyl radicals.

Much further work is required on both the physical aspects of the equipment and its configuration and the optimisation of the chemical conditions in order to define the best parameters for lignocellulose pre-treatment. This would constitue a fruitful area of further work but was outwith the scope of this current research.

5.3.7 Comparison of optimised processes for the conventional and ultrasonic pre-treatment of SG.

Results detailed in this chapter alongside those in chapter 4, give an optimised process methodology from the pre-treatment of SG using both conventional and ultrasonic techniques. Conversion efficiencies vary significantly between conventional acid/heat pre-treatment and ultrasonic methods detailed here (Table 5.25). Whilst ultrasound was shown to be effective in a combined ultrasound/chemical pre-treatment of SG, further development is required to increase its efficiency compared to conventional methods. The following chapter sought to further optimise the use of ultrasound in the extraction of sugar from SG, by evaluating the effect of ultrasound upon SG enzymatic digestion.

Table 5.25 Comparison of carbohydrate extraction efficiency between conventional and ultrasonic methods

SG type	Pre-treatment type	Actual carbohydrate extracted (g/100g SG)	% Conversion
DSG	120℃ Nitric acid	50.88	89.66
DSG	Ultrasound, O_3 & H_2O_2	34.31	60.46
BSG	120℃ Nitric acid	42.69	83.12
BSG	Ultrasound, O_3 & H_2O_2	27.22	52.99

5.4 Chapter summary

- The use of ultrasound alone is not effective in the pre-treatment of SG
- Ultrasound does not enhance acid or hydrogen peroxide pre-treatment of SG
- Ultrasound enhances ozonolysis of SG
- Ultrasound enhances combined ozone/ hydrogen peroxide pre-treatment of SG
- The use of ultrasound in the extraction of fermentable carbohydrate is not as effective as conventional methods.

Chapter 6

Effects of ultrasound on the enzymes involved in lignocellulose hydrolysis

6. Effects of ultrasound on the enzymes involved in lignocellulose hydrolysis.

6.1 Introduction

Investigation into the effects of ultrasonic irradiation upon the function of hydrolytic enzymes is a research area that is gaining increasing attention. Ultrasound has been shown to enhance the hydrolytic activity of a number of enzymes, including; invertase (Sakakibara, *et al*., 1996), pectinase (Yachmenev *et al*., 2001), alpha-amylase (Schmidt *et al*., 1987; Apar *et al*., 2006; Barton *et al*., 1996), glucoamylase (Schmidt *et al*., 1987), amyloglucosidase (Barton *et al*., 1996) and cellulase (Yachmenev *et al*., 2009; Condon *et al*., 2009).

Additionally, there are a number of studies that have sought to assess the use of ultrasound as a substrate pre-treatment prior to enzymolysis, rather than directly evaluating the effects of ultrasound upon the hydrolytic enzymes involved. Imai, Ikari and Suzuki (2004), found that when cellulose was pre-treated with ultrasound, there was enhanced sugar release during the subsequent enzymolysis. Khanal *et al*., (2007) reported similar observations in that pre-treatment of corn slurry prior to enzymolysis of starch with alpha-amylase and glucoamylase, caused an increase in the release of glucose compared to enzymolysis of non-sonicated corn slurry. It is also of note that ultrasound can be used as a tool for modelling hydrolytic enzymes. Reza and Buckin., (2011) reported on the use of high-resolution ultrasonic spectroscopy for real time analysis of β-glucosidase hydrolysis of cellobiose. However, none of these papers assessed the effects of ultrasound during enzymolysis of their respective substrates.

Until recently the effects of ultrasound upon the function of enzymes involved in the hydrolysis of lignocellulose was an area which had seen little attention. However, recent research has suggested that ultrasound has the potential to enhance the function of cellulase during the enzymolysis of cellulose (Yachmenev *et al*., 2009; Condon *et al*., 2009; Yasuda *et al*., 2010; Yoshimoto *et al*., 2004; Aliyu and Hepher, 2000; Wang *et al*., 2012).

Yachmenev *et al*., (2009) exposed cellulase enzymatic digestions of both corn stover and sugar cane bagasse to ultrasound at a frequency of 50 kHz. The researchers concluded that the application of ultrasound during enzymolysis increased the release of sugars from both types of lignocellulosic biomass. Condon *et al*., (2009) reported similar findings when they exposed cellulase enzymatic digestions of cotton to ultrasound with a frequency of 50 kHz. The researchers concluded that the application of ultrasound increased the release of glucose during the enzymolysis period. However it is of note that both Yachmenev *et al*., (2009) and Condon *et al*., (2009) failed to report the power output of their respective ultrasound generating systems.

Yoshimoto *et al*., (2004) reported similar findings when they exposed cellulase enzymatic digestions of waste paper to ultrasound at 20 kHz and 30 W. Ultrasound caused an increase in sugar release during the course of the enzymatic digestion. Aliyu and Hepher., (2000) exposed enzymatic digestions of cellulose powder and waste office paper to ultrasound at a frequency of 38 kHz and a power of 80W and concluded that ultrasound increased sugar release during enzymolysis of both substrates.

Yasuda *et al*., 2010 reported on the effects of ultrasound upon cellulase digestion of cellulose. The researchers evaluated three ultrasonic frequencies (20, 28 and 500 kHz) at varying ultrasonic powers (30, 40 and 50 W). It was reported that both 20 and 28 kHz ultrasound caused an increase in glucose release during the enzymolysis period. It is also of note that the magnitude of the increase in sugar release increase depended power input. The researchers also reported that exposure of enzymatic digestions to ultrasound with a frequency of 500 kHz actually caused a decrease in glucose release and that the magnitude of the decrease got larger as power increased.

The majority of these papers do little to assess the influence of varying ultrasonic frequency and output power upon enzyme function, usually concentrating upon a single ultrasonic frequency and fixed output power. Both of these factors are likely to influence the effects of ultrasonic irradiation upon enzyme function. Coupled with this, ultrasonic frequencies that have been studied are usually in the range of 20 – 50 kHz and little is known with regards to the effects of high frequency ultrasound (>500 kHz) upon enzyme function. Due to the fact that there has been little published with regards to the effects of high frequency ultrasound upon enzyme function research was conducted which sought to assess the impact of high frequency ultrasound upon the enzymes involved in lignocellulose hydrolysis. In addition the effects of ultrasonic power were also investigated in terms of its effect upon enzyme function.

6.2 Experimental approach

The experimental methodology employed assessed the effects of ultrasonic frequency and output power in terms of enzymatic digestion of micro-crystalline cellulose by cellulase and β – glucosidase. This gave an insight into the effects of ultrasound when both enzymes are employed together. In order to understand further the effects of ultrasound upon each of the enzymes individually, the experimental methodology was expanded to the digestion of cellulose without the presence of β –glucosidase and the hydrolysis of cellobiose by β –glucosidase. The final part of the research methodology involved assessing the effects of ultrasound upon Cellic (Ctec and Htec) hydrolysis of acid pre-treated SG. This was done with a view to assessing the potential of ultrasound to reduce required enzyme dosing rates during ligno-cellulose enzymolysis and thereby reduce the costs associated with enzymatic sugar extraction.

6.3 Results and discussion

6.3.1 Effects of ultrasound upon combined cellulase/ β-glucosidase hydrolysis of cellulose.

6.3.2.1 Influence of ultrasonic frequency

The first stage in this evaluation sought to assess the effects of ultrasonic frequency upon the combined hydrolysis of cellulose by cellulase and β – glucosidase. The experimental procedure involved exposing enzymatic digestions of cellulose powder to a variety of ultrasonic frequencies (584, 862, 998, 1174 kHz) with a standardised power output of 3 W and quantifying glucose release.

Ultrasound had a significant impact upon glucose release over the duration of the enzymatic digestion (Fig. 6.1). All of the ultrasonic frequencies utilised showed an increase in glucose release compared to the control sample (dosed with enzyme but not exposed to ultrasound), however this increase was highly dependent upon the frequency of the applied ultrasound. Enzymatic digestions exposed to ultrasound at 998 kHz displayed the greatest increase in glucose hydrolysis, followed by 1174 kHz, 862 kHz and 584 kHz. The exposure of enzymatic digestions to ultrasound also caused an increase in the total glucose yield during the course of the enzymatic digestion (Table 6.1) and again the increase was dependent upon the applied ultrasonic frequency.

The results suggest that the application of an ultrasonic field during the enzymolysis of cellulose increases the activity of the enzymes involved in the digestion and that the magnitude of the increase in enzyme activity is closely related to the frequency of the applied ultrasound. This is further

evidenced by the fact that the control sample (exposed to ultrasound but without enzyme dosing), displayed no glucose release throughout the duration of the enzymatic digestion. This suggests that the increase in glucose release is the result of ultrasound acting upon the enzymes rather than through the direct hydrolysis of cellulose. It is unclear from the data whether ultrasound is stimulating both cellulase and β – glucosidase, or one of the enzymes individually.

Table 6.1 – Influence of ultrasonic frequency upon glucose yield following 5h sonicated enzymolysis of cellulose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic frequency is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Frequency	**Total Glucose Yield (g/100g cellulose)**
584	13.76 (0.387)
862	15.39 (0.342)
998	17.95 (0.301)
1174	16.57 (0.099)
No ultrasound	12.79 (0.311)
No enzyme	0 (0)

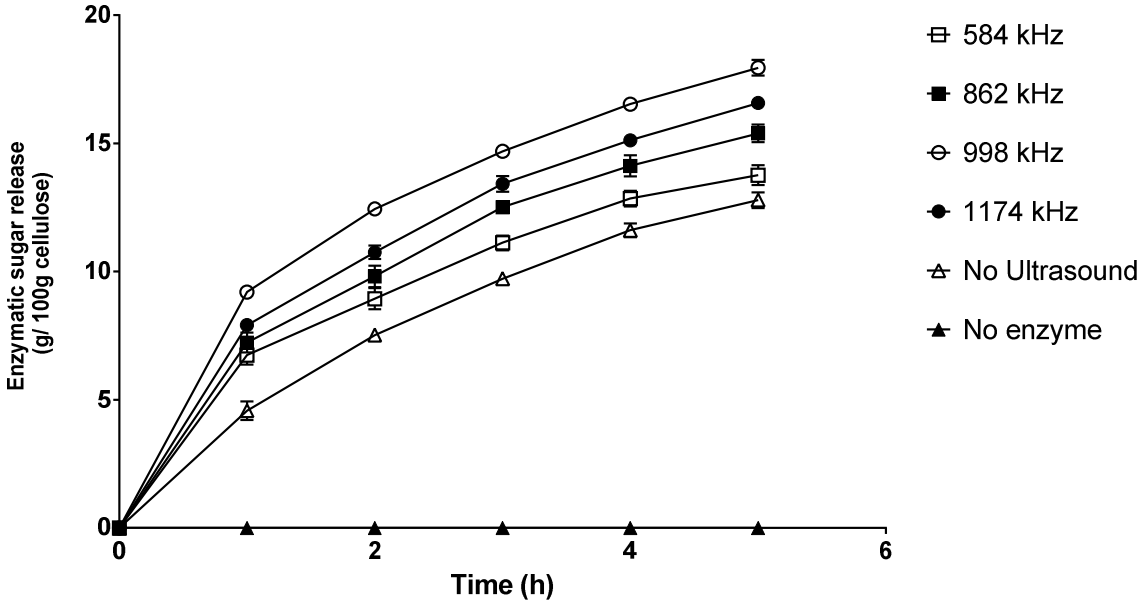


Fig. 6.1 Impact of ultrasonic frequency upon glucose release during combined cellulase and β-glucosidase enzymolysis of cellulose

(Results are means of triplicate treatment with error bars showing SD)

6.3.2.2 Influence of ultrasonic power

As has been shown above, the magnitude of the increase in the activity of cellulase and β–glucosidase, is closely related to ultrasonic frequency. Another parameter that is likely to be influential is ultrasonic output power and this was assessed in terms of its effect upon cellulase and β–glucosidase hydrolysis of cellulose by exposing enzymatic digestions to the optimal frequency identified previously (998 kHz), and varying the output power and periodically quantifying glucose release.

Ultrasound had a significant impact upon glucose release over the duration of the enzymatic digestion, with all of the ultrasonic powers employed showing an increase in glucose release compared to the control sample (dosed with enzyme but not exposed to ultrasound). However, the magnitude of this increase was highly dependent on the output power of the applied ultrasound (Fig. 6.2). Whilst all three of the ultrasonic output powers under study displayed an increase in glucose release, ultrasound at 3 W exhibited the greatest increase. The application of ultrasound at 6 W caused a decrease in glucose release compared to ultrasound at 3 W, however glucose release higher than that of the control not exposed to ultrasound was still observed. This indicates that increasing ultrasonic output power increases glucose release, however once an optimum power is achieved, increasing the power further starts to cause a decrease in glucose release.

Although not observed with the output powers used in this study, it is likely that increasing ultrasonic output power beyond a certain point will lead to an inhibitory effect upon enzyme function. The findings of Yasuda *et al*., (2010), suggested that the application of 500 kHz ultrasound at 30W to

cellulase, actually decreased enzyme function. However the data presented here indicates that this is likely to be attributed to the high output power rather than the frequency. Ultrasound at 500 kHz and a power of 30 W would be expected to generate significant levels of transient cavitation with the associated effects of cavitation bubble implosion which results in hydroxyl radical production. High frequency ultrasound such as that utilised by Yasuda *et al*., (2010) has been shown to generate higher levels of hydroxyl radicals than ultrasound at 20 kHz (Mason *et al*., 1994; Milne, Stewart and Bremner, 2012). It would appear likely that Yasuda *et al*., (2010) observed a decrease in enzyme activity due to the fact that their sonication system was generating levels of hydroxyl radicals that caused enzyme denaturation.

The high frequency low power ultrasound reported here would be expected to produce lower levels of hydroxyl radicals as low power ultrasound is generally believed to generate mainly stable cavitation and lower levels of transient cavitation (Laborde *et al*., 1998). Cavitation bubbles that are deemed to be stable oscillate at the applied ultrasonic frequency, whilst transient cavitation bubbles implode and result in the formation of hydroxyl radical production. The lower levels of transient cavitation produced by low power ultrasound ultimately leads to reduced hydroxyl radical formation.

The exposure of enzymatic digestions to ultrasound also caused a significant increase in the total glucose released during the course of the enzymatic digestion (Table 6.2) and again the increase was dependent upon the applied ultrasonic power. The application of ultrasound at 3 W appeared to be the optimum output power in accelerating the total glucose yield but

increasing the wattage to 6 W causing a reduction in yield. It was however noteworthy that all of the output powers assessed were typified by an increased glucose yield compared to the control.

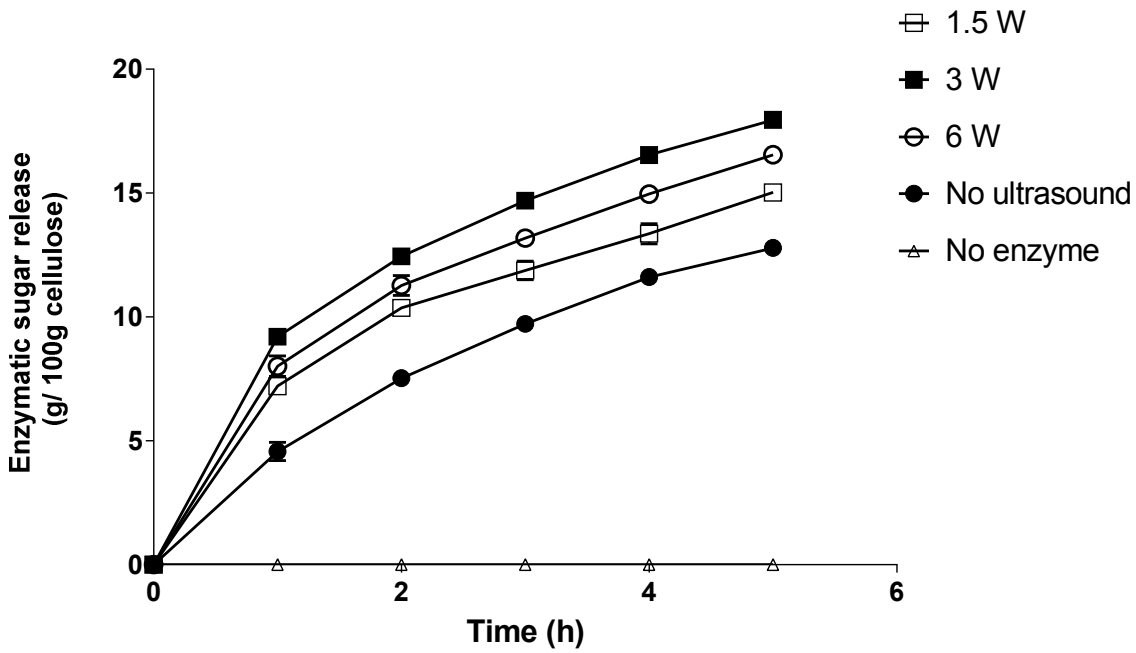


Fig. 6.2 Impact of ultrasonic power upon glucose release during combined cellulase and β-glucosidase enzymolysis of cellulose (Results are means of triplicate treatment with error bars showing SD)

Table 6.2 – Influence of ultrasonic output power upon glucose yield following 5h sonicated enzymolysis of cellulose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic power is significantly different - one way ANOVA, significance level, $p<0.05$

Ultrasonic power (W)	Total Glucose Yield (g/100g cellulose)
1.5	15.02 (0.311)
3	17.95 (0.301)
6	16.55 (0.333)
No ultrasound	12.79 (0.317)
No enzyme	0 (0)

6.3.2 Effects of ultrasound upon cellulase hydrolysis of cellulose

In order to attempt to determine if ultrasound was acting upon both cellulase and β – glucosidase or one of the enzymes individually, research was conducted that sought to assess the effects of ultrasound whilst hydrolysing their respective substrates separately. When used in combination, cellulase and β – glucosidase work in tandem to hydrolyse cellulose to glucose. Exocellulase cleaves individual cellulose chains at random points, thus generating a mixture of long chain polysaccharides alongside low levels of glucose. Endocellulase (comprising CBHI and CBHII) cleaves cellobiose subunits from the cellulose chain which is then subsequently hydrolysed to glucose by β – glucosidase. During research which assessed the effects of ultrasound upon both enzymes simultaneously, there was no cellobiose released as β – glucosidase loading was kept high, with the intention of avoiding end product inhibition of cellulase by cellobiose. This meant that cellobiose produced by cellulase was rapidly converted to glucose. However, when evaluating the effects of ultrasound upon cellulase hydrolysis of cellulose without the addition of β – glucosidase, a build up of cellobiose was observed. As such it was necessary to quantify both glucose and cellobiose.

6.3.2.1 Influence of ultrasonic frequency

The influence of ultrasonic frequency was studied in terms of its effects upon the function of cellulase during the hydrolysis of cellulose. As opposed to previous experiments where cellulose was digested with cellulase and β-glucosidase, the experimental strategy detailed here studied enzymolysis of cellulose with solely cellulase. This was done with a view to reducing the number of different enzymes within the reactor to gain a greater

understanding of the effects of ultrasound upon cellulase. The absence of β-glucosidase causes the sugar products glucose and cellobiose to be released as opposed to just glucose release observed previously. As discussed in chapter 1, the actions of endocellulase at random points along the cellulose fibre yields glucose, cellobiose and polysaccharides of varying length containing glucose subunits. Exocellulase then converts the longer chain polysaccharides to cellobiose. Consequently, hydrolysates were characterised for both glucose and cellobiose.

Ultrasound caused an increase in cellobiose hydrolysis from cellulose. As was observed in previous experiments this increase was highly dependent upon the frequency of the applied ultrasound (Fig. 6.3). All of the applied frequencies showed an increase in cellobiose release compared to the control sample which was loaded with cellulase and not exposed to ultrasound. This indicates that ultrasound accelerates the function of cellulase. A frequency of 998 kHz showed the largest increase in cellobiose release, followed by 1174, 862 and 584 kHz. All of the frequencies under study also showed an increased total cellobiose yield by the end of the enzymolysis period (Table 6.3). Enzymatic digestions that were exposed to ultrasound, along with the control sample not exposed to ultrasound, showed a limited increase in cellobiose concentration after 3 h, suggesting that as cellobiose reached a certain level it began to inhibit the hydrolysis. The control sample exposed to ultrasound with the addition of enzyme showed no sugar release throughout the hydrolysis period. This adds further evidence to previous observations that ultrasound was not directly hydrolysing cellulose.

Table 6.3 – Influence of ultrasonic frequency upon cellobiose yield following 5h sonicated enzymolysis of cellulose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic frequency is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Frequency (kHz)	**Total Cellobiose Yield (g/100g cellulose)**
584	4.81 (0.097)
862	5.09 (0.074)
998	5.65 (0.175)
1174	5.25 (0.107)
No ultrasound	4.09 (0.096)
No enzyme	0 (0)

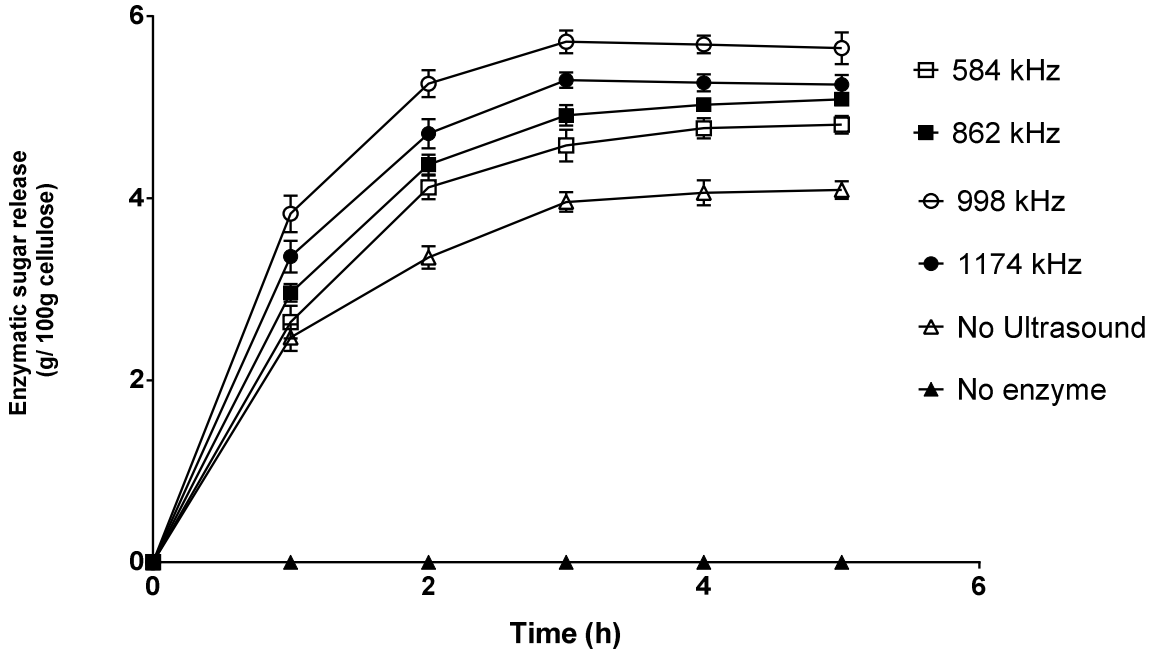


Fig. 6.3 Impact of ultrasonic frequency upon cellobiose release during cellulase enzymolysis of cellulose (Results are means of triplicate treatment with error bars showing SD)

Regarding glucose release, again all of the frequencies showed an increase compared to the controls (Fig. 6.4). This increase was linked to the frequency of the applied ultrasound with 998 kHz showing the greatest elevation, followed by 1174, 862 and 584 kHz. The increase in sugar hydrolysis resulted in a higher glucose yield after 5h sonication (Table 6.4). As was observed with cellobiose release, there was limited sugar release after 3 h, adding further evidence that the enzymatic digestions were inhibited when cellobiose levels reached a certain concentration.

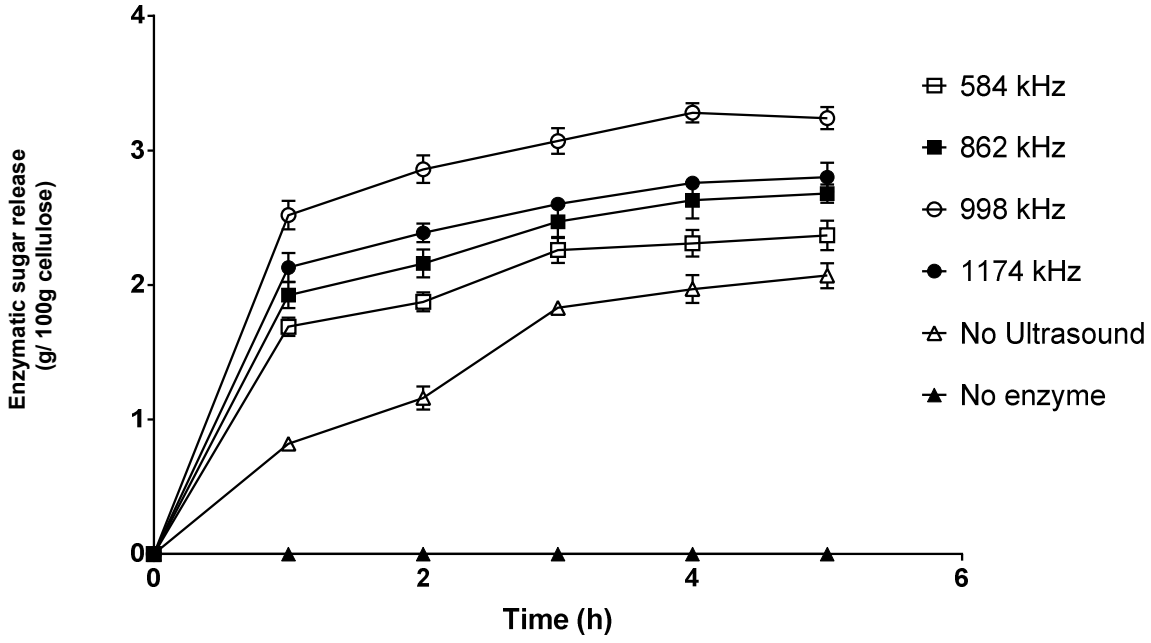


Fig. 6.4 Impact of ultrasonic frequency upon glucose release during cellulase enzymolysis of cellulose (Results are means of triplicate treatment with error bars showing SD)

Table 6.4 – Influence of ultrasonic frequency upon glucose yield following 5h sonicated enzymolysis of cellulose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic frequency is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Frequency (kHz)	**Total Glucose Yield (g/100g cellulose)**
584	2.37 (0.109)
862	2.68 (0.068)
998	3.24 (0.083)
1174	2.8 (0.107)
No ultrasound	2.07 (0.093)
No enzyme	0 (0)

6.3.2.2 Influence of ultrasonic power

Ultrasound increased glucose and cellobiose release over the duration of the enzymatic digestion. This was apparent with all of ultrasonic powers employed showing an increase in cellobiose (Fig. 6.5) and glucose (Fig 6.6) release compared to the control sample (dosed with enzyme but not exposed to ultrasound). This translated to an increased yield of cellobiose (Table 6.5) and glucose (Table 6.6) after 5h sonication, compared to the non-sonicated control. However, as was observed previously the magnitude of this increase was highly dependent on the output power of the applied ultrasound. Whilst all three of the ultrasonic output powers under study displayed an increase in glucose release, ultrasound at 3 W exhibited the greatest increase in glucose hydrolysis. The application of ultrasound at 6 W caused a decrease in glucose release compared to ultrasound at 3 W, however glucose release higher than that of the control not exposed to ultrasound was still observed. This suggests that increasing ultrasonic output power elevates glucose and

cellobiose release, however once an optimum power is achieved, increasing the power further starts to cause a decrease in glucose release.

Table 6.5 – Influence of ultrasonic power upon cellobiose yield following 5h sonicated enzymolysis of cellulose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic power is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Power (W)	Total Cellobiose Yield (g/100g cellulose)
1.5	5.19 (0.111)
3	5.65 (0.085)
6	5.21 (0.072)
No ultrasound	4.09 (0.096)
No enzyme	0 (0)

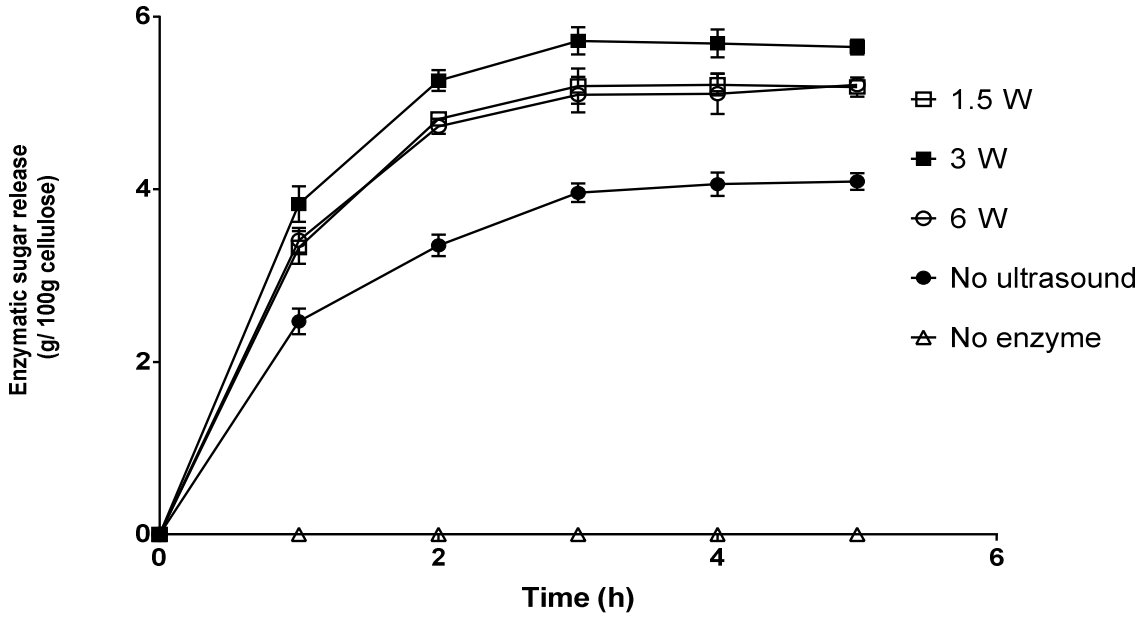


Fig. 6.5 Impact of ultrasonic power upon cellobiose release during cellulase enzymolysis of cellulose (Results are means of triplicate treatment with error bars showing SD)

Table 6.6 – Influence of ultrasonic power upon glucose yield following 5h enzymolysis of cellulose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic power is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Power (W)	Total Glucose Yield (g/100g cellulose)
1.5	2.59 (0.074)
3	3.24 (0.083)
6	2.47 (0.062)
No ultrasound	2.07 (0.093)
No enzyme	0 (0)

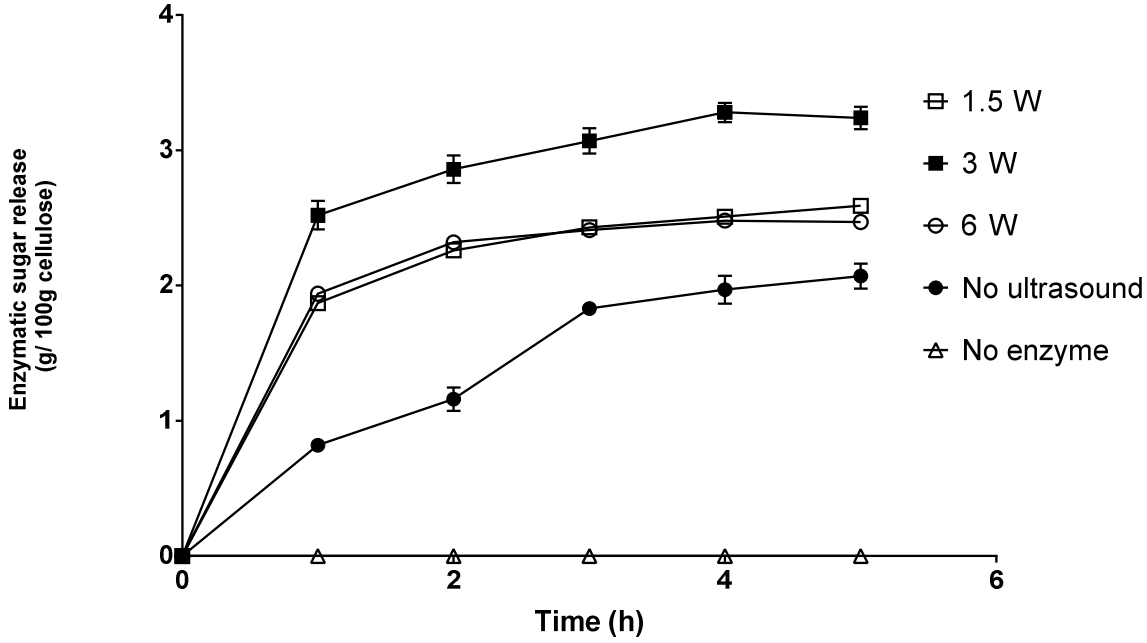


Fig. 6.6 Impact of ultrasonic power upon glucose release during cellulase enzymolysis of cellulose (Results are means of triplicate treatment with error bars showing SD)

6.3.3 Effects of ultrasound upon β-glucosidase hydrolysis of cellobiose

The final stage in determining whether ultrasound was acting upon cellulase or β –glucosidase, or indeed both enzymes, was to evaluate the effects of ultrasound upon β – glucosidase hydrolysis of cellobiose. The experimental procedure involved exposing enzymatic digestions of cellobiose solution to ultrasound and quantifying glucose release.

6.3.3.1 Influence of ultrasonic frequency

Exposure of enzymatic digestions to ultrasound caused an increase in enzymatic glucose liberation during enzymolysis of cellobiose by β – glucosidase, indicating that ultrasound enhances the function of β – glucosidase. As was observed in previous experiments, the magnitude of the increase sugar release was dependent upon the frequency of the ultrasound applied (Fig. 6.7), however the optimal frequency was different to that which was observed in previous experiments that involved the digestion of micro-crystalline cellulose. Ultrasound applied at 862 kHz showed the greatest increase, followed by 584 kHz, 998 kHz and 1174 kHz. In previous experiments the higher frequencies tended to display the greatest increase in sugars produced, however this appeared to be reversed during experiments assessing the effect of frequency variation upon enzymolysis of cellobiose. Cellulose is insoluble whereas cellobiose is soluble. Experiments that assessed the impact of ultrasound upon enzymolysis of cellobiose had no solid component within the reactor. This appears to have had an effect upon the optimal frequency for acceleration of the enzymatic digestion and is likely to be attributed to the fact that one system is homogeneous and the other is

heterogeneous and this will influence the transmission of ultrasonic energy in the system. Ultrasonically induced cavitation bubbles have been shown to form at the solid-liquid interface (Joseph *et al*., 2000, Peller *et al*., 2001). If solids are not present within the reactor the actions of cavitation would be spread throughout the reactor and this is likely to alter the most effective frequency in terms of accelerating enzyme function.

The application of ultrasound also caused an increase in the total glucose release during the 5 h enzymolysis period. Again all of the assessed frequencies showed higher total sugar yields, however there was a variation between the applied frequencies (Table 6.7).

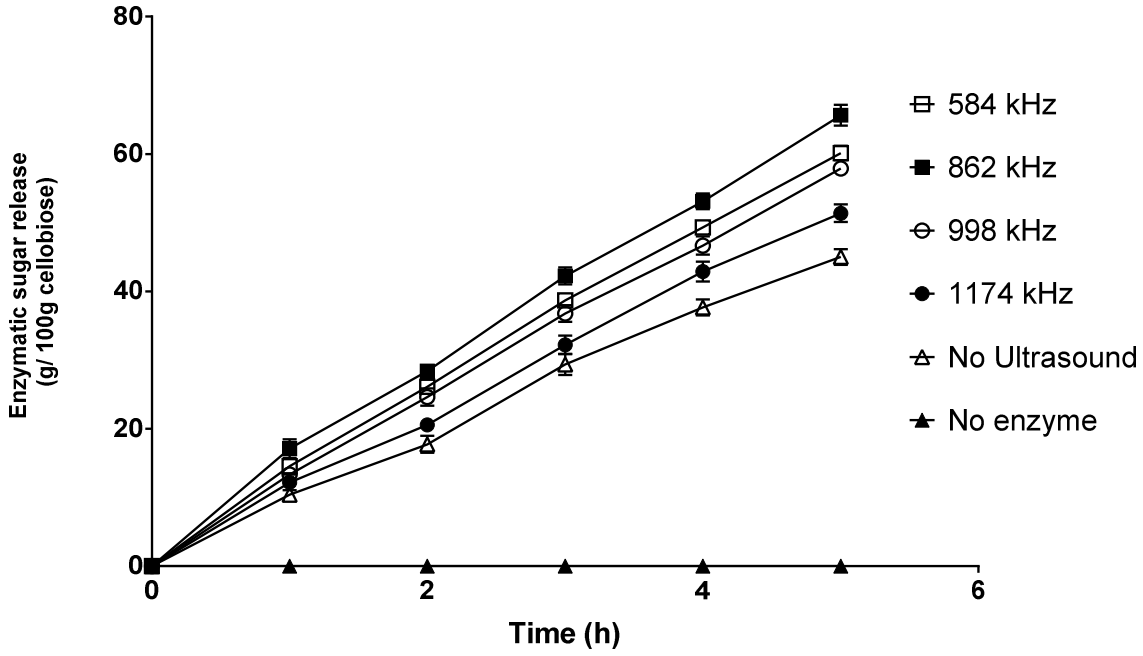


Fig. 6.7 Impact of ultrasonic frequency upon glucose release during β–glucosidase enzymolysis of cellobiose (Results are means of triplicate treatment with error bars showing SD)

Table 6.7 – Influence of ultrasonic frequency upon glucose yield following 5h sonicated enzymolysis of cellobiose (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic frequency is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Frequency (kHz)	**Total Glucose Yield (g/100g cellobiose)**
584	60.14 (1.056)
862	65.63 (1.521)
998	57.87 (1.014)
1174	51.39 (1.288)
No ultrasound	45.02 (1.14)
No enzyme	0 (0)

6.3.3.2 Influence of ultrasonic power

All three of the applied ultrasonic powers showed an elevation in glucose release compared to the control sample (dosed with enzyme but not exposed to ultrasound) during the hydrolysis of cellobiose by β – glucosidase (Fig. 6.8) and translated to an increase sugar yield at the cessation of the hydrolysis period (Table 6.8). However, as was observed previously the magnitude of this increase was highly dependent on the output power of the applied ultrasound. Whilst all three of the ultrasonic output powers under study displayed an increase in glucose release, ultrasound at 3 W exhibited the greatest increase in glucose hydrolysis. The application of ultrasound at 6 W caused a decrease in the glucose release compared to ultrasound at 3 or 1.5 W, however glucose release higher than that of the control not exposed to ultrasound was still observed. This provides further evidence that output power is a key parameter in determining the effects that ultrasound has upon hydrolytic enzymes.

Table 6.8 – Influence of ultrasonic power upon total glucose yield following 5h sonicated enzymolysis of cellobiose ((Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic power is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Power (W)	Total Glucose Yield (g/100g cellobiose)
1.5	56.65 (0.877)
3	65.63 (1.521)
6	54.9 (1.217)
No ultrasound	45.02 (1.145)
No enzyme	0 (0)

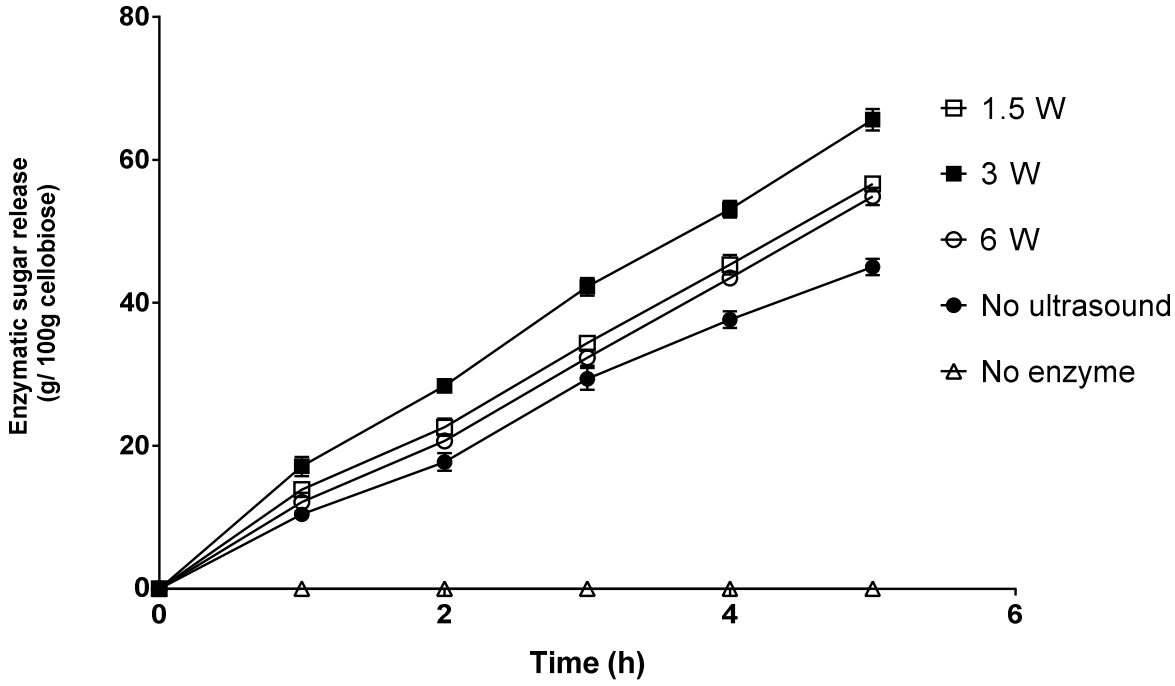


Fig. 6.8 Impact of ultrasonic power upon glucose release during β–glucosidase enzymolysis of cellobiose (Results are means of triplicate treatment with error bars showing SD)

Considering data from the effects of ultrasound upon combined cellulase and β – glucosidase hydrolysis of cellulose, cellulase hydrolysis of cellulose and β – glucosidase hydrolysis of cellobiose, it is clear that ultrasound accelerates the activity of both enzymes. Ultrasound has been shown to increase the sugar yield when the enzymes are employed individually or together. The frequency and power of the applied ultrasound appear to be the key parameters in determining the effect ultrasound has on both enzymes. Regarding ultrasonic power, an optimum of 3 W seemed to give the greatest increase in enzyme function across all of the experiments. In terms of the optimum frequency, this appeared to vary dependent upon the presence or absence of solids within the ultrasonic reactor.

6.3.4 Effects of ultrasound upon Cellic hydrolysis of spent grains

Research thus far showed that ultrasound increases the hydrolytic function of cellulase and β – glucosidase and that the magnitude of this increase is closely related to the frequency and power of the applied ultrasound. Whilst optimal ultrasonic power appeared to be 3 W for both cellulase and β – glucosidase, the optimal frequency tended to differ depending on whether or not there were solids present in the ultrasonic reactor. A frequency of 998 kHz showed the largest increase in sugar yield for hydrolysis of cellulose by cellulase and/or β – glucosidase, and 862 kHz for cellobiose by β - glucosidase. Reasons for the differences in the optimal ultrasonic frequency remain uncertain. In light of this, further research sought to assess the effects of ultrasonic frequency upon Cellic Ctec and Htec (cellulase, β – glucosidase and xylanase) digestion of BSG and DSG.

Of the four sources of spent grains assessed for compositional characterisation detailed in Chapter 3, two were selected for use in assessing the effects of ultrasound upon enzymolysis of SG. The samples selected for study were spent grains from the grain distillery (referred to as DSG in subsequent data) which were derived from 85% maize/15% malted barley and those from Bellhaven Brewery (referred to as BSG in subsequent data) which were derived from 100% barley malt.

Regarding DSG, ultrasound caused an increase in sugar release over the duration of the enzymatic digestion. This was apparent with all of the ultrasonic frequencies employed showing an increase in sugar release compared to the control sample (dosed with enzyme but not exposed to ultrasound). However, the magnitude of this increase was highly dependent on the frequency of the applied ultrasound (Fig. 6.9) where 998 kHz showed the greatest increase in sugars hydrolysed from DSG, followed by 862 kHz, 1174 kHz and 584 kHz. All of the frequencies under study showed an increased sugar yield for the major constituent sugars present in DSG hydrolysates (glucose, arabinose and xylose) (Table 6.9). These results provide further evidence that the exposure of a cellulose enzymatic digestion to ultrasonic irradiation, causes an increase in the hydrolytic efficiency of the enzymes involved in the digestion. Whilst not a focus of previous research in this chapter, the increased yield of the pentose sugars arabinose and xylose also suggests that ultrasound enhance the activity of xylanase.

Table 6.9 – Influence of ultrasound upon total sugar yield following 24h sonicated enzymolysis of DSG (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic frequency is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Frequency (kHz)	Sugar Yield (g/100g SG)			
	Glucose	Arabinose	Xylose	Total
584	17.54 (0.425)	1.02 (0.103)	4.13 (0.128)	22.69 (0.614)
862	18.57 (0.378)	1.94 (0.087)	7.14 (0.283)	27.65 (0.698)
998	19.32 (0.191)	2.63 (0.76)	7.74 (0.098)	29.69 (0.358)
1174	17.51 (0.303)	1.47 (0.109)	4.86 (0.267)	23.84 (0.614)
No ultrasound	14.27 (0.101)	0.67 (0.035)	2.85 (0.024)	17.79 (0.089)
No enzyme	0.41 (0.026)	0.62 (0.047)	0.77 (0.034)	1.8 (0.091)

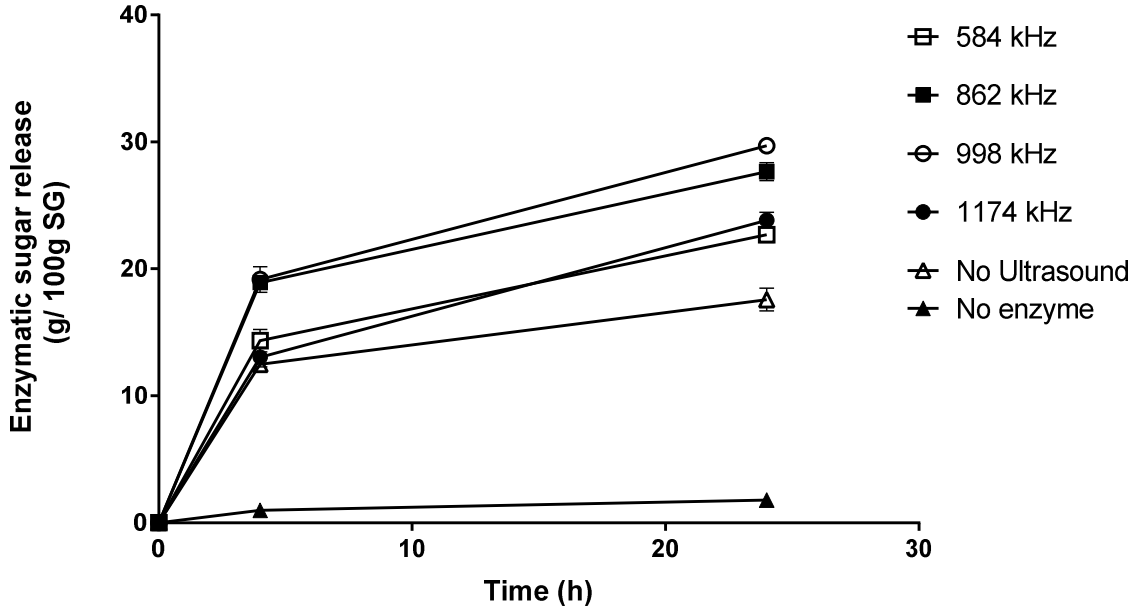


Fig 6.9 Influence of ultrasound upon sugar release during enzymolysis of DSG (Results are means of triplicate treatment with error bars showing SD)

Ultrasound also had a significant impact upon sugar release from BSG during the enzymatic digestion, causing an increase compared to the control sample. However, the magnitude of this increase was, once more, very highly dependent on the frequency of the applied ultrasound (Fig. 6.10). A frequency of 1174 kHz showed the greatest increase in sugar release from BSG during enzymolysis, followed by 998 kHz, 584 kHz and 862 kHz. This translated to an increased release of the three main hydrolysate sugars (glucose, xylose and arabinose) and a larger sugar yield following 24h sonication(Table 6.10). This provides further evidence for the hypothesis that ultrasound enhances the hydrolytic efficiency of cellulase and β – glucosidase and xylanase.

Table 6.10 – Influence of ultrasonic power upon total sugar yield following 24h sonicated enzymolysis of BSG (Results are means of triplicate treatment with SD in parenthesis. Each ultrasonic frequency is significantly different - one way ANOVA, significance level, $p<0.05$)

Ultrasonic Frequency (kHz)	**Sugar Yield (g/100g SG)**			
	Glucose	**Arabinose**	**Xylose**	**Total**
584	9.08 (0.106)	1.29 (0.067)	3.93 (0.112)	14.3 (0.335)
862	8.31 (0.469)	0.92 (0.121)	2.76 (0.233)	11.99 (0.611)
998	11.87 (0.316)	1.33 (0.091)	3.82 (0.037)	17.02 (0.402)
1174	12.44 (0.397)	1.8 (0.189)	4.81 (0.061)	19.05 (0.511)
No ultrasound	7.86 (0.152)	0.62 (0.177)	2.15 (0.088)	10.63 (0.371)
No enzyme	0.7 (0.079)	0.39 (0.052)	0.43 (0.031)	1.52 (0.187)

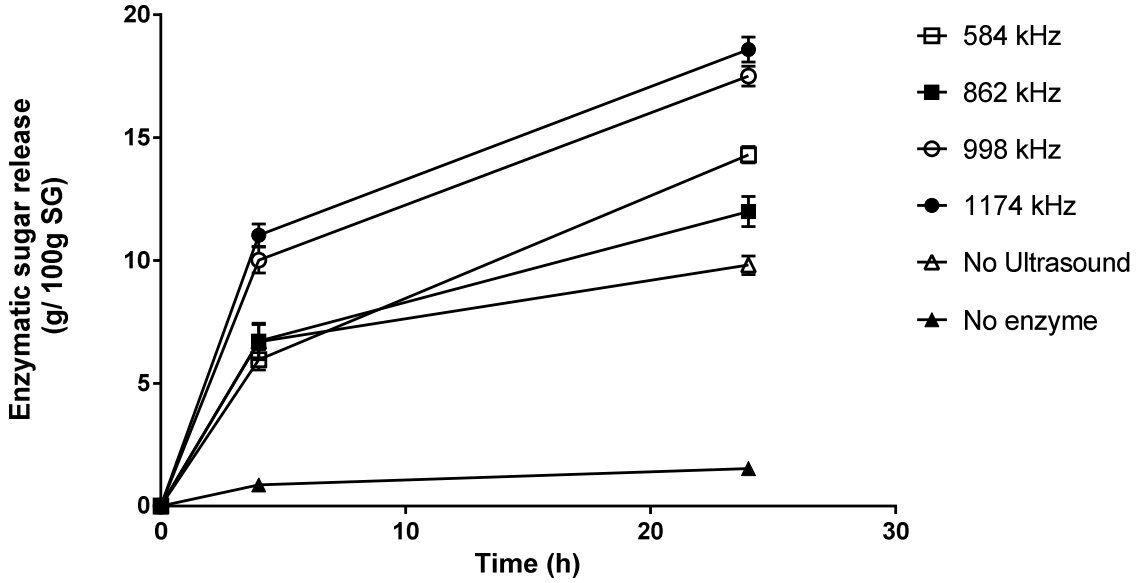


Fig 6.10 Influence of ultrasound upon sugar release during enzymolysis of DSG (Results are means of triplicate treatment with error bars showing SD)

6.4 Conclusions

Results have shown that the effects of ultrasound upon enzyme function are closely related to the frequency and power of the applied ultrasound. Research conducted using either cellulose or cellobiose indicated that the optimal frequency for accelerating enzyme function was dependent upon the characteristics of the sonication medium i.e. the presence or absence of solids within the reactor. A frequency of 998 kHz was optimal for enzymolysis of cellulose and 862 kHz for cellobiose. However, data from SG experiments expanded this, indicating that the composition of the solids within the reactor also plays a role. The optimal frequencies for accelerating enzymatic digestions of DSG and BSG, were 998 kHz and 1174 kHz, respectively. The reasons for the difference in optimal frequencies is unclear, however it is of

note that the DSG and BSG used in this study were maize and malted barley based, respectively. It is seems likely that differences are attributable to differences in compositional characteristics between the SG types (e.g. total carbohydrate, lignin or protein).

Relatively little is known about the exact mechanism by which ultrasound acts to increase hydrolytic enzyme function. Various mechanisms have been postulated, including that ultrasound: decreases the liquid solid diffusion layer, enhance emulsification, generates micro-streaming, alters surface potential or accelerates molecule transport (Kwiatkowska *et al*., 2011). Ultimately all of these potential mechanisms can be thought of as increasing mixing within the ultrasonic reactor thereby enhancing interaction between enzyme proteins and their associated substrate. Unfortunately not enough work in this area has been done to be definitive and none of the published literature proves any of the proposed mechanisms conclusively.

There are various reports which speculate on the mechanisms by which ultrasound appears to enhance the function of hydrolytic enzymes. Yachmenev *et al*. (2009) proposed a simplified mechanism of reaction for cellulase enzymes involved in the hydrolysis of cellulose (Fig. 6.11). The process starts with the diffusion of the enzyme macromolecule from the aqueous phase to the surface of the cellulose fibre. The enzyme then adsorbs onto the fibre surface and beings hydrolysing sugar subunits from the fibre. The final stage in the process involves the diffusion of the sugar products to the aqueous phase. Both transfer of enzyme macromolecules to the substrate surface along with transfer of the sugar products are facilitated by diffusion. Yachmenev *et al*. (2009) concluded that as enzyme

marcromolecules are large they have low diffusion rates which impede the rate at which sugars are hydrolysed from cellulose. As such the researchers hypothesis that ultrasound increases the activity of cellulase by increasing molecule transport and consequently the rate at which cellulase adsorbs onto the cellulose fibre.

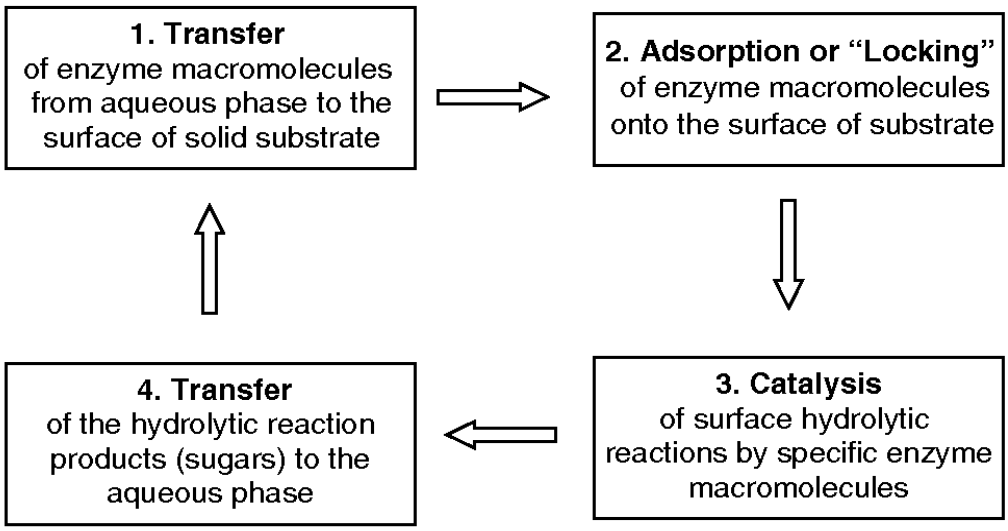


Fig. 6.11 Process steps in the hydrolysis of cellulose by cellulase (adapted from Yachmenev *et al*., 2009).

Condon *et al*., (2009) hypothesised a similar mechanism by which ultrasound increases cellulase activity by enhancing diffusion of enzyme macromolecules towards the surface of the substrate. However the researchers went a step further and hypothesised a specific mechanism by which ultrasound actually enhances molecule transport. In a system which contains solid particles suspended within a liquid that is being mixed via mechanical agitation there are a number of liquid layers that concentrically surround the solid particle (Fig. 6.12). The first layer immediately at the solid liquid interface is motionless due to the fact that mechanical agitation is

ineffective in stirring this particular layer (Condon *et al*., 2009). Mechanical agitation can however affect the outer layers and agitation velocities increase as the layer increases in distance from the suspended particle.

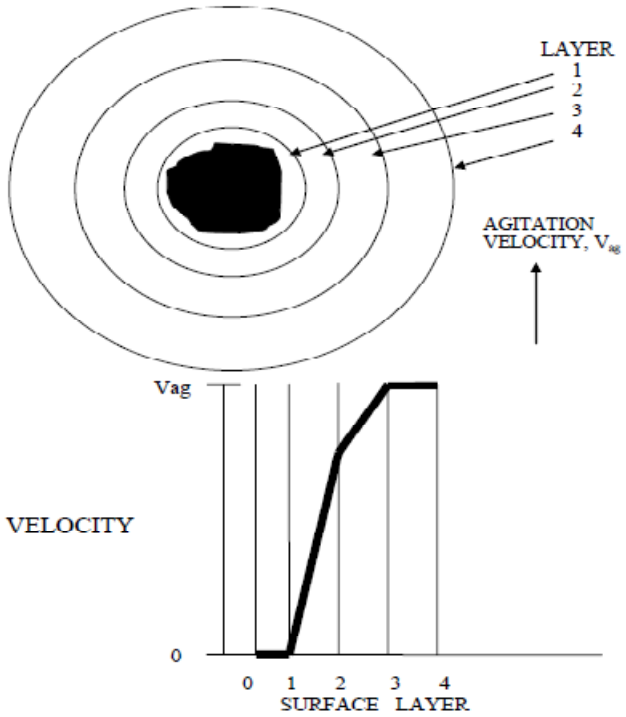


Fig. 6.12 Overview of the velocities of the liquid layers surrounding a particle suspended within a liquid medium (adapted from Condon *et al*., 2009)

Due to the inability of mechanical agitation to affect mixing at the solid-liquid boundary, the only process available to transport cellulase enzyme macromolecules to the cellulose surface is simple diffusion. Diffusion is known to be highly inefficient in transporting large protein molecules such as cellulase (Condon *et al*., 2009) and is one of the limiting factors in cellulase hydrolysis of cellulose. As described in chapter 5 the actions of ultrasound upon a liquid medium produce cavitation bubbles within the medium. If solids are present within the liquid, cavitation bubbles form at the solid-liquid interface (Joseph *et al*., 2000, Peller *et al*., 2001). For this

reason Condon *et al*., (2009) concluded that the actions of cavitation bubbles within the layer of liquid immediately at the solid-liquid interface provided a means other than diffusion for transporting enzyme macromolecules to the surface of the substrate. This then results in enhanced adsorption of enzymes to the substrate surface and an enhancement of the hydrolytic efficiency of cellulase.

Other literature reports rarely discuss in depth the potential mechanisms by which ultrasound increased sugar release during enzymolysis of cellulose. However most appear to be in agreement with the ideas postulated by Condon *et al*., (2009) and Yachmenev *et al*. (2009), in that ultrasound appears to increase the rate at which cellulose diffuses towards and adsorbs onto the cellulose fibre (Yasuda *et al*., 2010; Yoshimoto *et al*., 2004; Aliyu and Hepher, 2000). However, Yoshimoto *et al*., (2004) proposed an additional mechanism whereby the action of cavitation bubbles at the liquid solid not only aid the transport of cellulase towards to cellulose surface but also facilitates the removal of the sugar products from the active site of the enzyme. The researchers hypothesised that this decreases the time before the enzyme can re-interact with substrate.

Taken as a whole, the data detailed in this chapter has shown that ultrasound enhances the function of cellulase, β – glucosidase and xylanase, resulting in an increased sugar yield. The results are largely in agreement with other published studies which assess the effects of ultrasound upon the enzymes involved in lignocellulose hydrolysis. However results reported here give additional insight into the fact that ultrasound at higher frequencies can also increase enzyme function as well as the lower frequencies reported in

other studies. Whilst it is not possible to deduce an exact mechanism by which ultrasound is increasing sugar release during enzymolysis, it appears likely that it can be attributable to the mechanisms proposed by Yachmenev et al. (2009) and Condon et al., (2009). These centre upon the actions of ultrasonic cavitation bubbles in reducing the solid-liquid diffusion layer and accelerating molecule transport. Both of these factors would be expected to increase the rate at which the enzyme adsorbs to its substrate.

However these potential mechanisms must also be viewed in the context of the results published by Imai, Ikari and Suzuki (2004) and Khanal *et al*., (2007). Both of these studies found that pre-treating cellulose powder with ultrasound prior to enzymolysis resulted in increased sugar yield suggesting that ultrasound can also increase the susceptibility of cellulose powder to enzymatic digestion. It would appear that ultrasound can increase sugar release during enzymatic digestion through a combination of mechanisms, those being the physical effects of ultrasound in increasing the porosity of cellulose alongside the increased molecular transport of the enzyme towards its target substrate. However, much more work is required, probably on simpler enzymatic systems, to fully delineate the positive effects caused by ultrasound. Many possible mechanisms exist and this is probably a rich area for future research.

Results presented in this chapter have shown that ultrasound has the potential to significantly reduce enzyme loading rates employed during the enzymolysis of lignocelluloses. As discussed in chapter 1, enzymes represent one of the major costs associated with bioethanol derived from

lignocellulosic feed stocks and ultrasound has the potential to reduce these costs.

6.5 Summary

- Ultrasound increases the hydrolytic activity of cellulase and β – glucosidase during the celullolysis of cellulose.
- Ultrasound increases the hydrolytic activity of Cellic Ctec and Htec during enzymolysis of spent grains.
- The magnitude of the increase in enzyme activity is closely linked to the frequency and power of the applied ultrasound.
- The presence of solids within the reaction medium has an effect upon which frequency causes the greatest increase in enzyme activity
- The use of ultrasound during the enzymolysis of lignocellulose has the potential to reduce enzyme loading levels.

Chapter 7

Fermentation of spent grain hydrolysates

7. Fermentation of spent grain hydrolysates

7.1 Introduction

The use of yeast in the fermentation of sugars to ethanol is one of the oldest biotechnological practices employed by man, usually in the production of potable alcoholic beverages. *Saccharomyces cerevisiae* is the yeast of choice in traditional starch and sugar based fermentations as it is efficient in providing high ethanol yields as well as displaying excellent ethanol tolerance (Olsson and Hahn – Hagerdal, 1996). However, the fermentation of lignocellulose derived hydrolysates presents new challenges, both in terms of the sugars to be fermented as well as the presence of fermentation inhibitors.

The major component sugars in lignocellulose hydrolysates are glucose, arabinose and xylose. Whilst *S.cerevisiae* can ferment the former, it does not possess the ability to ferment the pentose sugars, unless genetically modified (Walfridsson *et al*., 1995; Jin and Jeffries, 2004; Verho *et al*., 2003; Verho *et al*., 2004). Nevertheless, various strains of wild-type yeast species possess the capability to ferment both the hexose and pentose sugars present within lignocellulose hydrolysates. These yeasts include: *Pichia Stipitis* (now re-named *Scheffersomyces stipitis), Kluyveromyces marxianus, Candida shehatae* and *Pachysolen tannophilus*. Various studies have reported successful fermentation of lignocellulose hydrolysates using pentose fermenting yeasts (Preez, Bosch and Prior, 1986; White *et al*., 2008; Perogo *et al*., 1990).

7.2 Experimental approach

The aim of this Chapter was to evaluate a number of yeast species in terms of their ability to ferment the sugars present within spent grain hydrolysates. This was done with a view to assessing the fermentability of hydrolysates produced using optimised acid/heat pre-treatment (detailed in Chapter 4) of SG from the grain whisky distillery (DSG) and Bellhaven Brewery (BSG). It was envisaged that this could serve as a future benchmark for assessing the fermentability of hydrolysates produced with ultrasonic pre-treatment. The yeasts studied were *S.cerevisiae, P.stipitis, K.marxianus, P. tannophilus and C. Shehatae*. Hydrolysates were fermented for 124h and sugar utilisation, ethanol production, yeast growth and viability were determined periodically.

7.3 Results and discussion

7.3.1 Fermentation of DSG hydrolysates

7.3.1.1 Sugar utilisation

There was a variation between yeast species in terms of sugar usage during fermentation of DSG hydrolysates (Table 7.1). Each of the fermentation media (hydrolysates) prepared from 10% w/v DSG contained: 24.75, 20.75 and 9.25gL^{-1} of glucose, xylose and arabinose, respectively. All of the yeasts under study utilised all of glucose present within the fermentation medium. However, there was a variation in the rate of glucose utilisation between yeast species. *K.marxianus* displayed the greatest rate of glucose utilisation, with all the hydrolysate glucose being consumed within 24 h. This was in contrast to *P.stipitis* which took 71h to consume glucose. *P.tannophilus*, *S.cerevisiae* and *C.shehatae* showed complete glucose utilisation after 51, 51 and 31h, respectively. As expected, *S.cerevisiae* did not consume xylose

or arabinose, due to its inability to metabolise pentose sugars. Xylose consumption varied between the pentose utilising species under study, with *K.marxianus* showing the greatest xylose consumption with usage of 13.38gL^{-1}. This compared to xylose utilisation in the other pentose utilising species of: 8.88gL^{-1} for *P.tannophilus*, 4.51gL^{-1} for *C.shehatae* and 8.5gL^{-1} for *P.stipitis*. None of the pentose utilising species showed any arabinose consumption. Thesis findings indicate that DSG presents a challenging fermentation medium for all of the yeasts under study showing relatively poor sugar usage and is likely to be attributed to the presence of fermentation inhibitors within DSG hydrolysate. As discussed in Chapter 4, the pre-treatment of lignocellulose leads to the formation of a variety of fermentation inhibitors.

Table 7.1 Yeast sugar usage during fermentation of DSG hydrolysates

Yeast	Sugar utilisation (gL^{-1})		
	Glucose	**Xylose**	**Arabinose**
S. cerevisiae	24.75	0	0
K. marxianus	24.75	13.38	0
C. shehatae	24.75	4.51	0
P. stipitis	24.75	8.5	0
P. tannophilus	24.75	8.88	0

7.3.1.2 Yeast growth and viability

During fermentation of DSG hydrolysates, all of the yeasts showed relatively poor rates of growth (Fig. 7.1). Cells were inoculated at an initial cellular density of 10 x 10^6 cells/ml. At the end of fermentation *P.tannophilus*

displayed the largest increase in cell number, reaching a final cell density of 178 x 10^6 cells/ml. This compared with final cellular densities of 104 x 10^6, 124 x 10^6, 138 x 10^6 and 131 x 10^6 cells/ml for *S.cerevisiae, P.stipitis, K.marxianus and C. shehatae*, respectively.

The five yeast species displayed relatively high viability levels at the end of fermentation (Fig. 7.2). This is with the exception of *S.cerevisiae* which showed a drop in viability to 58.26%. This compared to 84.28, 82.36, 91.54 and 78.32% for *P.stipitis, K.marxianus, P. tannophilus and C. shehatae*, respectively. It is likely that the drop in *S.cerevisiae* viability can be attributed to a lack of a suitable carbon source following glucose depletion.

7.3.1.3 Ethanol production

There was a variation in ethanol production between the yeast species tested, with ethanol production being relatively low in all fermentations (Fig. 7.3). From fermentations of hydrolysates prepared from 10% w/v DSG, *K.marxianus* yielded the greatest ethanol production, with a final ethanol concentration of 1.63 % (v/v). This compared to 1.33, 0.85, 1.13 and 0.72% (v/v) for *S.cerevisiae, P.stipitis, K.marxianus, P. tannophilus and C. shehatae*, respectively. Such yields would not be considered commercially viable for large-scale ethanol production purposes.

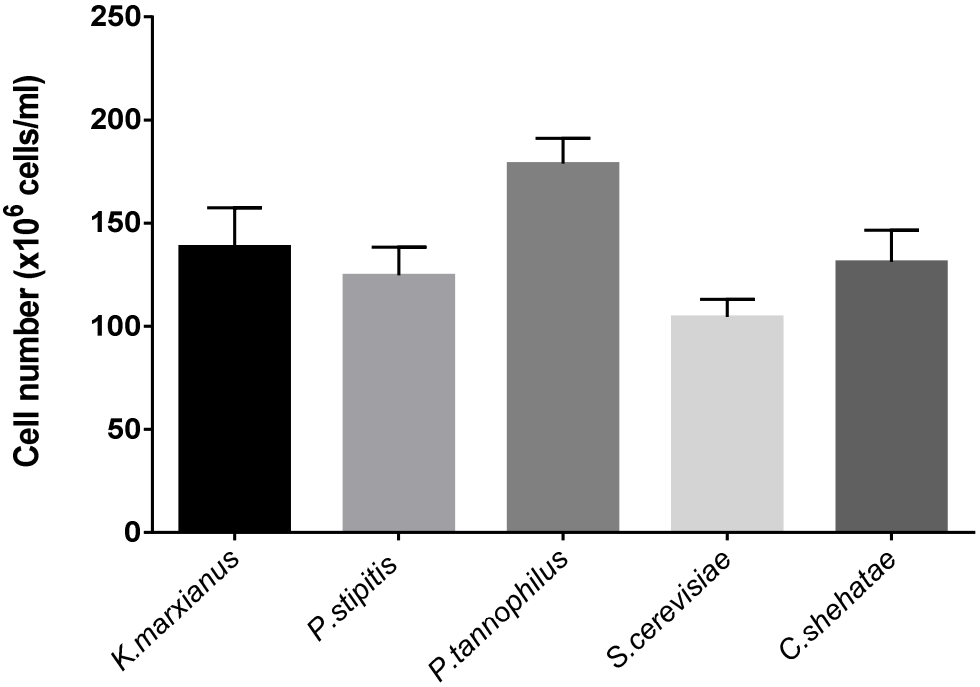


Fig. 7.1 Yeast cellular densities at the end of DSG hydrolysate fermentation

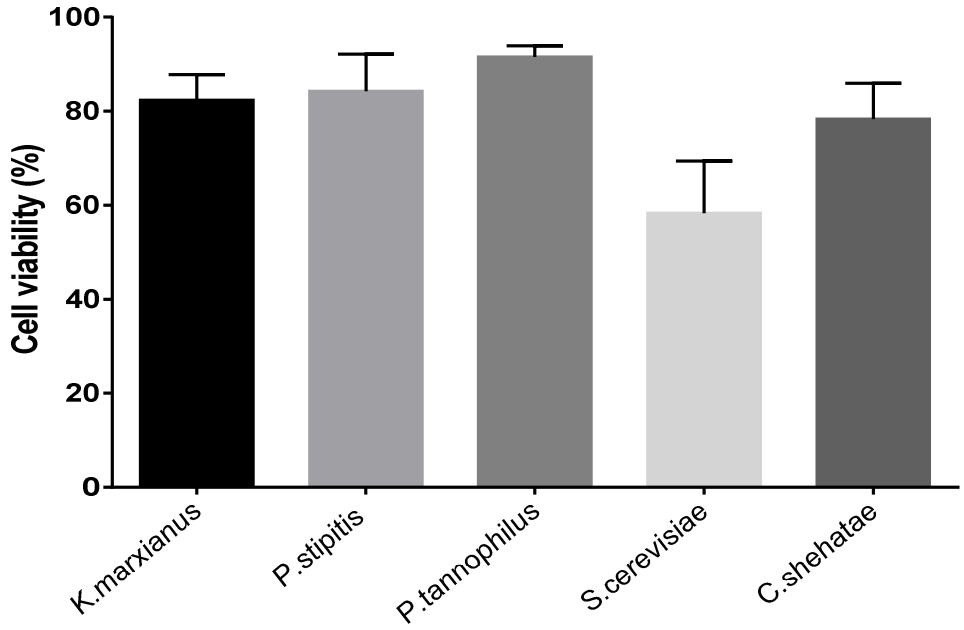


Fig. 7.2 Yeast viability at the end of DSG hydrolysate fermentation

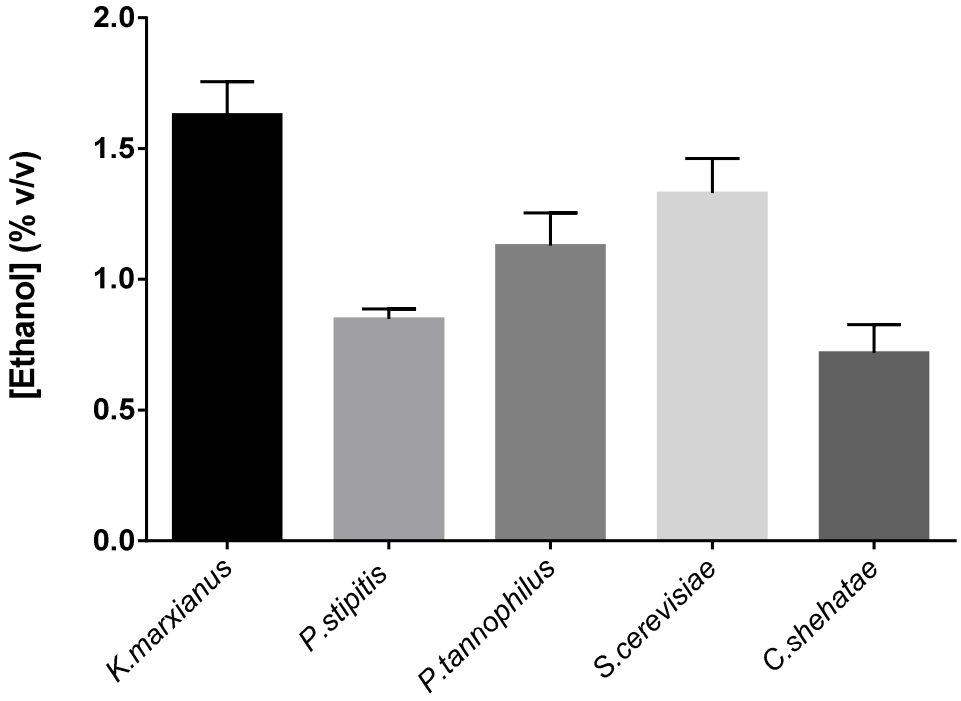


Fig. 7.3 Ethanol yield from fermentation of DSG hydrolysates

7.3.2 Fermentation of BSG hydrolysates

7.3.2.1 Sugar utilisation

Each of the fermentation media (hydrolysates) prepared from 10% w/v DSG contained: 20.25, 16.38 and 9.38gL^{-1} of glucose, xylose and arabinose, respectively. During fermentation of BSG hydrolysate all five of the yeast species under study, consumed all of the available hydrolysate glucose. As was observed in fermentation of DSG hydrolysates, there was a variation in the rate of glucose usage between species. *S.cerevisiae* and *C.shehatae* displayed the greatest rate, with both species showing complete glucose utilisation by 51 h. This compared to *K.marxianus*, *P.stipitis* and

P.tannophilus which showed complete glucose utilisation by 71, 71 and 103 h, respectively.

As expected, *S.cerevisiae* did not use either arabinose or xylose following glucose depletion. Whilst xylose utilisation varied between the species that possess the ability to metabolise pentose sugars, *K.marxianus* showed the greatest consumption, with a total consumption 14.75gL^{-1}. This compared to *C.shehatae*, *P.tannophilus* and *P. stipitis*, which showed xylose usage of 9.1, 7.75 and 10.13 gL^{-1}, respectively. As was observed in fermentation of DSG hydrolysates, none of the yeast species consumed any arabinose during fermentation of BSG hydrolysates.

Table 7.2 Yeast sugar usage during fermentation of BSG hydrolysates

Yeast	Sugar utilisation (gL^{-1})		
	Glucose	**Xylose**	**Arabinose**
S. cerevisiae	20.25	0	0
K. marxianus	20.25	14.75	0
C. shehatae	20.25	9.1	0
P. stipitis	20.25	7.75	0
P. tannophilus	20.25	10.13	0

7.3.2.2 Yeast growth and viability

As was observed during fermentation of DSG hydrolysates, there was a relatively low level of yeast growth observed during fermentation of BSG hydrolysates (Fig. 7.4). At the end of fermentation *P.tannophilus* displayed the largest increase in cell number, reaching a final cell density of 202 x 10^6 cells/ml. This compared with final cellular densities of 167 x 10^6, 142 x 10^6,

127 x 10^6 and 134 x 10^6 cells/ml for *S.cerevisiae, P.stipitis, K.marxianus and C. shehatae*, respectively.

The majority of yeasts under study displayed relatively high viability levels at the end of fermentation (Fig. 7.5). This is with the exception of *S.cerevisiae* which showed a drop in viability to 67.85%. This compared to 86.97, 84.57, 76.38 and 89.41% for *P.stipitis, K.marxianus, P. tannophilus and C. Shehatae*, respectively. As discussed previously the drop in *S.cerevisiae* viability is likely to be attributable to the lack of a usable source of sugar following glucose depletion. However, there is also the possibility that fermentation inhibitors present in SG hydrolysate, is resulting in a decrease in viability. White *et al*. (2008) reported a similar observation when fermenting SG hydrolysates using the same yeast species.

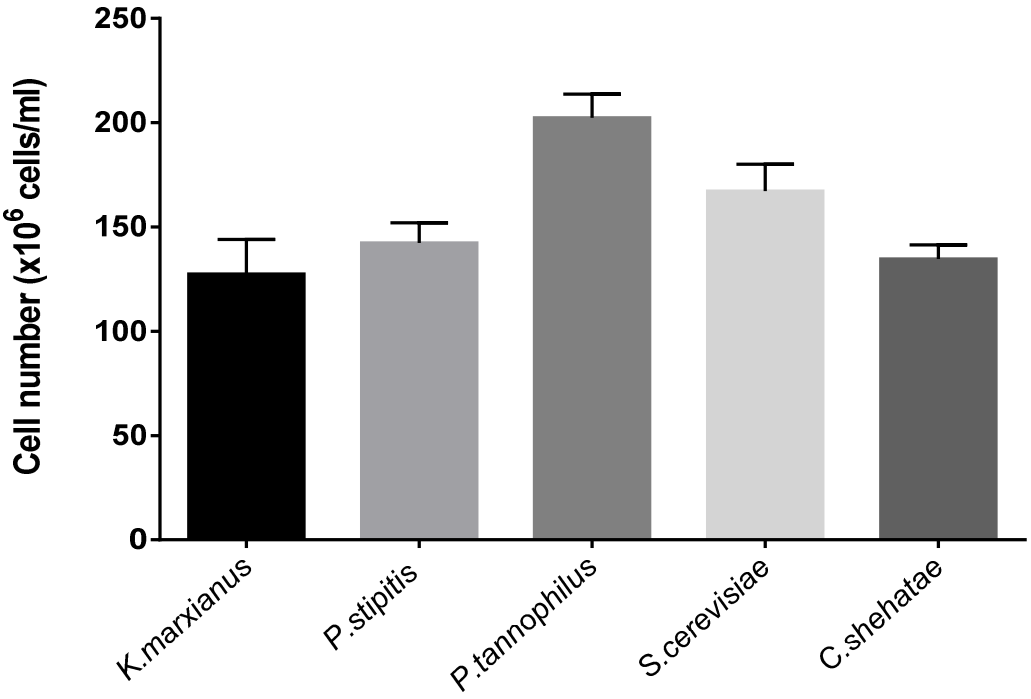


Fig 7.4 Yeast cellular densities at the end of BSG hydrolysate fermentation

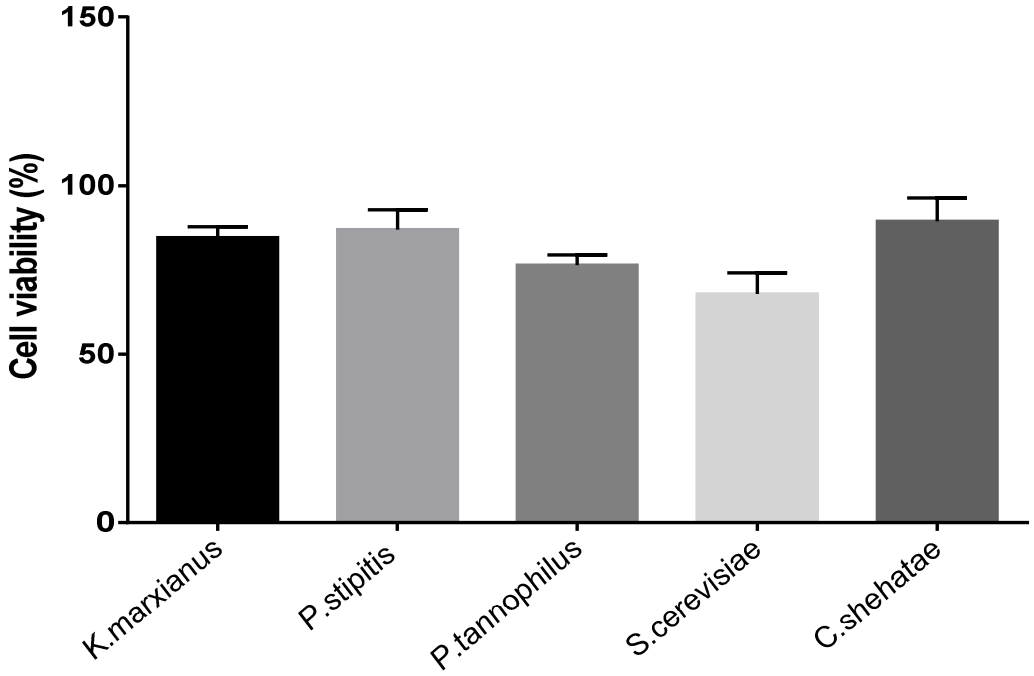


Fig 7.5 Yeast viability at the end of BSG hydrolysate fermentation

7.3.2.3 Ethanol production

Ethanol yields from fermentation of BSG hydrolysates were of a similar to those observed during fermentation of DSG hydrolysates, in that ethanol production was relatively low. There was a variation in ethanol production between the yeast species under study (Fig. 7.6). From fermentations of hydrolysates prepared from 10% w/v BSG, *K.marxianus* yielded the greatest magnitude of ethanol production, with a final ethanol concentration of 1.46 % (v/v). This compared to 1.28, 1.13, 0.87 and 0.96% (v/v) for *S.cerevisiae, P.stipitis, K.marxianus, P. tannophilus and C. shehatae*, respectively. As with the ethanol yields obtained from fermentations of DSG hydrolysates, these would not be considered commercially viable for distillation to bioethanol.

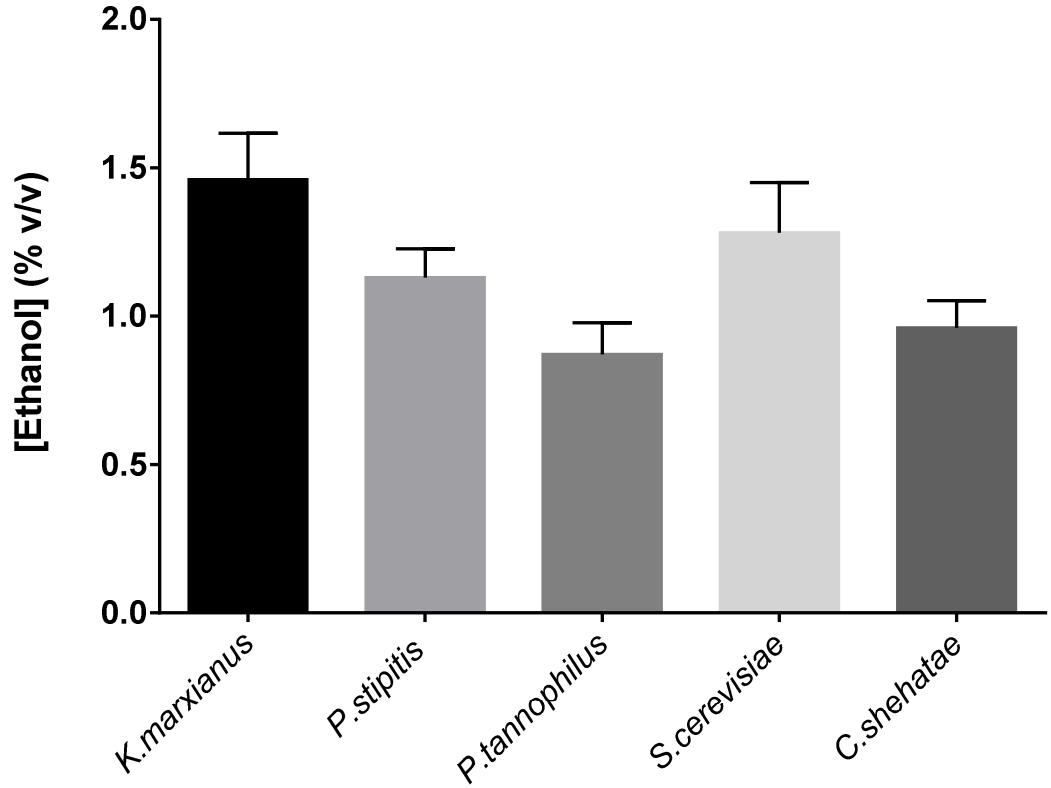


Fig. 7.6 Ethanol yield from fermentation of BSG hydrolysates

7.3.3 Concluding discussion

During fermentation of DSG hydrolysates, *P. tannophilus* showed relatively poor ethanol yields compared to *S.cerevisiae* or *K.marxianus* . However, it also showed the highest levels of growth during the course of the fermentation. This suggests that rather than directing sugar metabolism towards fermentation, it was directed towards cell division. *K.marxianus* showed the highest level of ethanol production and pentose utilisation, but lower growth than *P. tannophilus*. This indicates that sugar consumption was directed towards fermentation rather than cell division. *P.stipitis* fermentations were characterised by relatively high levels of sugar consumption, however growth and ethanol production were relatively low.

Fermentation of BSG hydrolysates, were similar to those of DSG hydrolysates, in that they were characterised by poor sugar utilisation and relatively low ethanol yields. *P. Tannophilus* showed the lowest level of ethanol production, however as observed previously this was coupled with the highest rates of growth. This provides further evidence that sugars consumed by *P. tannophilus* are being directed towards cell division as opposed to ethanol production. Again, *K.marxianus* showed the highest ethanol production and sugar utlisation, alongside lower rates of growth compared to *P. tannophilus*. This lends itself to the point made previously, in that *K.marxianus* is directing sugar utilisation towards fermentation rather than growth.

Considering the data from fermentation of SG hydrolysates, it is clear that all of the fermentations were typified by relatively poor sugar utilisation alongside poor ethanol yields. It is of note that none of the yeasts utilised the arabinose present within either BSG or DSG hydrolysates and this can most likely be attributed to fact that pentose metabolising yeast species which can ferment significant quantities of arabinose are rare (Dien *et al*., 1996). Whilst not assessed directly, it appears likely that the poor fermentation performance onbserved in this study is due to the presence of fermentation inhibitors produced during pre-treatment and enzymolysis of SG. Fermentation inhibitors are likely to lead to slow fermentations with poor sugar utilisation and low ethanol yields rather than a complete cessation of fermentation activity (Jonsson, Alriksson and Nilvebrant, 2013).

At high temperatures xylose and arabinose degrade to furfural and glucose degrades to form 5-hydroxymethy furfural (HMF) (Palmqvist and

Hahn – Hagerdal, 1999). Furfural and HMF can further degrade yielding formic acid, whilst HMF can also form levulinic acid. Heat degradation of lignin yields a variety of phenolic compounds including vanillic acid, coniferyl and sinapyl alcohol. In addition to fermentation inhibitors formed by lignin and sugar degradation the break-down of hemicellulose results in the formation of acetic acid from hydrolysed acetyl side chains.

The aliphatic acids (acetic, formic and levulinic) present within lignocellulose hydrolysates have all been shown to be inhibitory to fermenting yeast (Jonsson, Alriksson and Nilvebrant, 2013). The primary mechanism by which they are thought to act is through the diffusion of undissociated acids over the cell membrane. Once inside the cell acids dissociate due to the neutral pH within the cell. This results in an increase in intracellular pH and can result in cell death (Pampulha and Loureiro-Diad, 1989). The mechanisms of fermentation inhibition by phenolic compounds produced by lignin degradation are highly variable depending upon specific functional groups (Ando *et al.*, 1986). In the case of many phenolic compounds the exact mechanism of fermentation inhibition is unknown however it is thought that they may interfere with the cell membrane and change its protein to lipid ratio thereby affecting its function (Jonsson, Alriksson and Nilvebrant, 2013).

Fermentation of hydrolysates produced through ultrasonic pre-treatment were not investigated in this thesis, but it is envisaged that with further optimisation of ultrasonic pretreatments of spent grains lower concentrations of chemical inhibitors in resultant hydrolysates would be achieved, due to the lower temperature employed during pre-treatment. This

may then be expected to result in better yeast fermentation performance in subsequent spent grain bioconversions to ethanol. These are areas worthy of possible future investigation, especially with regard to potential energy savings.

7.4 Summary

- Fermentations with selected yeast species showed relatively low sugar utilisation and low ethanol yields. It appears likely this is due to the presence of fermentation inhibitors.
- Of the yeasts assessed, *K.marxianus* was the most effective in fermenting DSG and BSG hydrolysates, both in terms of sugar utilisation and ethanol yield.
- As expected *S.cerevisiae* was unable to ferment the pentose sugars within SG hydrolysates.
- *P. tannophilus* appeared to direct sugar consumption to cell division rather than ethanol production.
- Further optimisation of the use of ultrasonic pre-treatment in the production of spent grain hydrolysates, may be expected to improve yeast fermentation performance due to lower levels of chemical inhibitors.

Chapter 8

Concluding discussion

8. Concluding discussion

The overall aim of the research presented in this thesis was to assess the potential for applying ultrasound in the process stages involved in the bioconversion of brewer's and distiller's spent grains to bioethanol. The use of ultrasound in both the pre-treatment and enzymolysis of SG was evaluated. In order to achieve this it was firstly necessary to characterise different spent grain samples and to extract fermentable carbohydrates from the lignocellulosic fractions using conventional methods. This provided a benchmark with which to compare ultrasonic pre-treatment and enzymolysis techniques.

8.1 Spent grain compositional analysis

Compositional characterisation of both types of SG was accomplished successfully, in terms of: klason lignin, carbohydrate, protein, moisture and ash. As had been shown in earlier studies (White *et al*., 2008), the results showed that SG is a rich source of potentially fermentable carbohydrate. The compositional characteristics of the SG utilised in this study were shown to be broadly similar to those reported in previous work (Russ *et al*., 2005; Mussatto *et al*., 2004; Mussato *et al*., 2005; Robertson *et al*., 2010; Niemi *et al*., 2012; Santos *et al*., 2003; Carvalheiro *et al*., 2004; Kanauchi *et al*. 2001), with variations being attributable to differing cereal grains used in production (e.g. maize and malted barley) and also differences in processing present between breweries and distilleries. For example DSG, obtained from a grain distillery were predominantly maize-based, whilst BSG obtained from an ale brewery were all-malt. Additionally, the maximum potentially extractable

carbohydrate from each of the SG sources was ascertained, and this enabled the assessment of conversion efficiencies of the pre-treatment techniques detailed in Chapter 5. The maximum extractable carbohydrate was found to be 56.75 and 51.36 g $(100g\ SG)^{-1}$ for DSG and BSG, respectively. Differences in carbohydrate content are likely to attributable to the fact that DSG was maize based and BSG was barley-malt based.

8.2 Conventional techniques for the pre-treatment and enzymolysis of spent grains

As has been found by previous studies (White *et al*., 2008; Du *et al*., 2010; Shuai *et al*., 2010; Romani *et al*, 2010), the present research showed that the use of acid was highly effective in the pre-treatment of lignocelluloses, particularly 1M HNO_3 when combined with heat treatment (120℃ for 20min). This was then followed by enzymatic digestion for 24h with an enzyme loading of 600 EGU and 100 FXU of Cellic Ctec and Cellic Htec, respectively. Carbohydrate conversion efficiencies using the optimised process were found to be 89.66 and 83.12% for DSG and BSG, respectively. This showed that relatively straightforward thermochemical pre-treatment followed by enzymatic cellulolysis was very effective in deconstructing spent grains to free sugars.

8.3 The use of ultrasound in the pre-treatment of spent grains

Disappointingly, it was found that ultrasound was not effective in the pre-treatment of either BSG or DSG. Samples exposed to ultrasound showed similar levels of hydrolysate sugars to the non-sonicated controls. This was the case with all of the ultrasonic powers and residence times employed

during the study. This contrasted with previously published work that found ultrasound to be effective in the pre-treatment of ligno-cellulose from sources other than SG (Yu *et al*., 2008; Sul'man et al., 2011). Yu *et al*., (2008) reported that when rice hull was exposed to ultrasound at 50 kHz and 250 W it caused a higher release of sugars during enzymatic digestion compared to untreated samples. Sulman *et al*., (2011) reported that exposure or sunflower husk to ultrasound at 30 kHz rendered the biomass amenable to further hydrolysis.

As discussed in Chapter 5, there are two potential mechanisms by which ultrasound can be thought of as acting upon lignocellulose. Firstly, the physical effects of cavitation bubble implosion upon the surface of the biomass results in an increase in the porosity of the lignocellulose matrix. Secondly, the action of ultrasound within an aqueous medium generates hydroxyl radicals though the dissociation of water. Hydroxyl radicals are thought of as being able to degrade lignin through the oxidation of C-H bonds contained within lignin subunits (Ek, Gierer and Jansbo, 1989) and are now believed to one of the mechanisms by which wood decay fungi degrade the lignocellulose structural matrix (Wood, 1994; Blanchette, 1995; Hyde and Wood, 1997; Hammel *et al*., 2002).

The results reported here suggesting that ultrasound is ineffective in the pre-treatment of SG are likely to be attributable to two potential factors. Those being; 1) the applied parameters of frequency and power were not successful in the generation of sufficiently violent cavitation bubbles to physically affect the biomass; and 2) the lack of sufficiently energetic cavitation resulted in production of hydroxyl radicals in quantities insufficient

to affect lignin degradation. The differences between the results reported here and reports within the primary literature are likely to be the result of the fact that these studies used higher ultrasonic frequencies (30 kHz - Sul'man et al., 2011, 40 kHz - Yu *et al*., 2008) and different sources of lignocellulose (Sunflower husk - Sul'man et al., 2011, rice hull - Yu *et al*., 2008) than those utilised in this study. Differing ultrasonic frequencies and the biomass type exposed to ultrasound are likely to have a role in how effective ultrasound is in the pre-treatment in lignocellulose.

The level of hydroxyl radical production by the action of ultrasound upon aqueous systems has been shown to increase with frequency (Mason *et al*., 1994; Milne, Stewart and Bremner, 2012). This suggests that the experimental methodology employed by both Sulman *et al*., (2011) and Yu *et al*., (2008), where ultrasound was applied to lignocellulose at frequencies of 30 kHz and 40 kHz respectively, resulted in enhanced hydroxyl radical formation compared to the ultrasonic frequency reported here (20 kHz). In addition to ultrasonic frequency, lignin contents of various types of biomass are likely to play a role in how effective a specific pre-treatment technique is likely to be. Higher lignin levels will render biomass more resistant to pre-treatment due to the fact there are simply larger relative amounts of lignin to degrade and access the polysaccharide beneath. Sulman *et al*., (2011) exposed sunflower husk to ultrasonic irradiation whilst Yu *et al*. (2008) used rice hull. Both of these sources of biomass have been shown to have similar levels of lignin to that of SG (~20% w/w – Demirbas, 2008). Therefore, it would appear likely that the ineffective use of ultrasound in the pre-treatment of SG reported here is the result of reduced hydroxyl radical production due

to the lower frequency ultrasound used in this study compared to frequencies used by Sulman *et al*., (2011) and Yu *et al*. (2008).

Ultrasound was also shown to have no effect upon the effectiveness of SG pre-treatment using acid or hydrogen peroxide. Ultrasound has been shown to generate hydroxyl radicals through the dissociation of hydrogen peroxide (Huang *et al*., 1993). Consequently, ultrasound may be expected to improve the effectiveness of hydrogen peroxide pre-treatment of SG through the formation of hydroxyl radicals produced by dissociation of hydrogen peroxide or water present within the reactor. However the results presented here suggest that ultrasound does not increase the effectiveness of hydrogen peroxide pre-treatment of lignocellulose. Whilst it is not possible to give a definitive reason for this being the case, it appears likely that ultrasound at the applied parameters was unsuccessful in generating sufficient quantities of hydroxyl radicals through either dissociation of hydrogen peroxide or water. This may be attributable to hydrogen peroxide concentrations within the reactor being too low.

It was found that ultrasound increased the effectiveness of ozonolysis of SG and increased the levels of hydrolysate sugars following enzymolysis. Sugars released during pre-treatment were found to comprise solely glucose. This is likely to be the result of residual starch hydrolysis as there was no release in pentose sugars, making it unlikely that the glucose observed was from cellulose or hemi-cellulose hydrolysis. This was observed in SG exposed to ultrasound and O_3 and those treated with solely O_3. However, during enzymatic digestion a substantial increase in hydrolysate sugars was observed. This suggests that O_3 was effective in degrading the lignin fraction

within SG and rendered the cellulose and hemi-cellulose fractions amenable to enzymatic hydrolysis. Ultrasound was found to enhance this process, presumably by augmenting the attack on the substrate by reactive oxygen species (ROS). It appears likely that combined physical effect of ultrasound and ozonolysis, is more effective in lignin degradation than the solely ozonolysis.

The data presented does not lend itself to a definitive conclusion as to why ultrasound enhances ozonolysis of SG. However it would appear likely that this can be attributed to enhanced hydroxyl radical formation within the reactor. Ultrasound has been shown to generate hydroxyl radicals through dissociation of ozone (Gogate and Pandit, 2004). Therefore pre-treatment with ultrasound and ozone can be thought of as being able to generate hydroxyl radicals through two mechanisms, those being through the ultrasonic dissociation of ozone or water present within the reactor. As such it would appear likely that the enhanced effectiveness of pre-treatment with ultrasound and ozone compared to solely ozone is likely to be due to the actions of hydroxyl radicals and associated degradation of lignin.

The reaction of ozone and hydrogen peroxide (peroxone reaction) is one type of advanced oxidation process that has shown to be effective in the production of hydroxyl radicals (Vogelpohl and Kim, 2004) and its use has long been established in waste-water treatment (Camel and Bermond, 1998; Acero and von Guten, 2000). As discussed in Chapter 5, the mechanisms of the reaction between ozone and hydrogen peroxide are well understood and ultimately result in the production of hydroxyl radicals through the consumption of ozone and hydrogen peroxide (Forni *et al*., 1982; Sehested

et al., 1982; Buhler *et al*., 1984). Ultrasound has been shown to generate hydroxyl radicals through dissociation of either ozone or hydrogen peroxide (Huang et al., 1993; Gogate and Pandit, 2004). Although it has not been proven conclusively within the field of sono-chemistry that ultrasound can enhance hydroxyl radical production during the reaction between ozone and hydrogen peroxide, it would appear likely that it provides a more direct route to hydroxyl radical production through dissociation of ozone and/or hydrogen peroxide.

Ultrasound was also shown to enhance pre-treatment of SG using a combination of O_3 and H_2O_2. Samples pre-treated with these oxidants showed a relatively high release of pentose sugars (arabinose and xylose). As these sugars are the main constituents of hemi-cellulose, this indicates that the use of O_3 and H_2O_2 is effective in the hydrolysis of hemi-cellulose. Whilst pre-treatment with solely O_3 did not result in a high level of pentose sugar release during pre-treatment, the addition of H_2O_2 seemed to improve this. This is evidenced by the fact that SG pre-treated with ultrasound, O_3 and H_2O_2 showed an increased yield of pentose sugars during pre-treatment compared to that pre-treated with only O_3 and H_2O_2. It appears likely that the presence of O_3 and H_2O_2 created a synergistic effect between the oxidising effects of O_3 and increased free radical production from ultrasonic degradation of H_2O_2. This resulted in increased hemi-cellulose hydrolysis.

During enzymolysis, SG that had been pre-treated with O_3 and H_2O_2 showed a relatively high degree of enzymatic sugar release. This indicates that there was a relatively a degree of lignin degradation. However, samples exposed to ultrasound showed a higher level of enzymatic sugar release

compared to the non-sonicated control, suggesting that ultrasound improves lignin degradation during pre-treatment of SG with O_3 and H_2O_2. The mechanism by which ultrasound increases lignin degradation during combined O_3 and H_2O_2 is uncertain. However, it is likely that the specificity of O_3 to hydrolyse double bonds, alongside improved ultrasonic free radical production during sonication from the presence H_2O_2, lead to relatively effective degradation of lignin and hemi-cellulose.

Although hydroxyl radical production was not measured in this study, the increased effectiveness of pre-treatment of SG with ultrasound, ozone and hydrogen peroxide compared to pre-treatment with ozone and hydrogen peroxide appears likely to be attributable to increased hydroxyl radical production within the ultrasonic reactor. The mechanism by which ultrasound enhances free radical production is likely to be the result of the dissociation of ozone and/or hydrogen peroxide (Huang et al., 1993; Gogate and Pandit, 2004). Hydroxyl radicals are thought of as being able to degrade lignin and polysaccharides through the oxidation of C-H bonds contained within subunits (Ek, Gierer and Jansbo, 1989) and could be the mechanism for increased lignin and hemicellulose degradation during pre-treatment of SG with ultrasound.

8.4 Influence of ultrasound upon the enzymes involved in spent grain hydrolysis.

Yachmenev *et al*., (2009) exposed cellulase enzymatic digestions of both corn stover and sugar cane bagasse to ultrasound at a frequency of 50 kHz. The researchers concluded that the application of ultrasound during enzymolysis increased the release of sugars from both types of

lignocellulosic biomass. Condon *et al*., (2009) reported similar findings when they exposed cellulase enzymatic digestions of cotton to ultrasound with a frequency of 50 kHz. Yoshimoto *et al*., (2004) reported that when they exposed cellulase enzymatic digestions of waste paper to ultrasound at 20 kHz and 30 W. Aliyu and Hepher., (2000) exposed enzymatic digestions of cellulose powder and waste office paper to ultrasound at a frequency of 38 kHz and a power of 80W and concluded that ultrasound increased sugar release during enzymolysis of both substrates. Yasuda *et al*., 2010 reported on the effects of ultrasound upon cellulase digestion of cellulose. The researchers evaluated three ultrasonic frequencies (20, 28 and 500 kHz) at varying ultrasonic powers (30, 40 and 50 W). It was reported that both 20 and 28 kHz ultrasound caused an increase in glucose release during the enzymolysis period.

As discussed in Chapter 6, ultrasound was shown to enhance the activities of cellulase and β–glucosidase during enzymatic digestion of crystalline cellulose by both enzymes, and also β–glucosidase digestion of cellobiose. This was expanded to show that the application of ultrasound enhances sugar release during Cellic digestion of SG. The research conducted indicated that the magnitude of the increase in the hydrolytic enzyme function was dependent on parameters such as ultrasonic frequency and output power, as well as the presence of solids within the ultrasonic reactor. Optimal ultrasonic frequencies were found to differ dependent on the solubility of the substrate.

Results are in agreement with the findings of other work, in that ultrasound enhances cellulase activity (Yachmenev *et al*., 2009; Condon *et*

al., 2009; Yoshimoto *et al*., 2004; Aliyu and Hepher. 2000). However, these findings are in contrast with other research studies with regards to the optimal frequency for enzyme acceleration. For example, Yasuda *et al*. (2010) found that the application of ultrasound at 500 kHz actually decreased cellulase activity, contrary to finding presented in this thesis. However, it appears likely that high ultrasonic power (30W) is the cause of enzyme inhibition. Research conducted during this project found that 3W was the optimal ultrasonic power for increasing enzyme function. As yet, it is unclear as to the mechanism by which ultrasound increases enzyme activity. However, various potential mechanisms have been suggested, including that ultrasound: decreases the liquid solid diffusion layer, enhances emulsification, generates micro-streaming or accelerating molecule transport. It is conceivable that ultrasound increases enzyme activity through enhancing the rate at which the enzyme protein interacts with its substrate (Kwiatkowska *et al*., 2011).

Both transfer of enzyme macromolecules to the substrate surface along with transfer of the sugar products are facilitated by diffusion. Yachmenev *et al*. (2009) postulated that as enzyme marcromolecules are large they have low diffusion rates which impede the rate at which sugars are hydrolysed from cellulose. As such the researchers hypothesised that ultrasound increases the activity of cellulase by increasing molecule transport and consequently the rate at which cellulase adsorbs onto the cellulose fibre. Condon *et al*., (2009) hypothesised a similar mechanism by which ultrasound increases cellulase activity by enhancing diffusion of enzyme macromolecules towards the surface of the substrate. However they

also proposed that as mechanical agitation cannot affect the layer of liquid immediately at the solid-liquid boundary and that cavitation bubbles tend to form in this area, the actions of cavitation bubbles in this region offers enzyme proteins another mechanism by which to transport to the surface of the substrate other than diffusion. This ultimately results in enhanced binding of cellulase to the cellulose fibre

Taken as a whole, the results detailed in Chapter 6 have shown that ultrasound enhances the function of cellulase, β – glucosidase and xylanase. The results are largely in agreement with other published studies which assess the effects of ultrasound upon the enzymes involved in lignocellulose hydrolysis (Yachmenev *et al*., 2009; Condon *et al*., 2009; Yasuda *et al*., 2010; Yoshimoto *et al*., 2004; Aliyu and Hepher, 2000; Wang *et al*., 2012). However results reported here give additional insight into the fact that ultrasound at higher frequencies can also increase enzyme function as well as the lower frequencies reported in other studies. Whilst it is not possible to deduce an exact mechanism by which ultrasound is increasing sugar release during enzymolysis, it appears likely that it can be attributable to the mechanisms proposed by Yachmenev et al. (2009) and Condon et al., (2009). These centre upon the actions of ultrasonic cavitation bubbles in reducing the solid-liquid diffusion layer and accelerating molecule transport. Both of these factors would be expected to increase the rate at which the enzyme adsorbs to its substrate.

8.5 Significance of research findings

The project has shown pent grains to be a valuable commodity and that they have the potential to be utilised in ways other than for cattle feed. Novel uses of the co-products generated by the brewing and distilling industries is of current topical importance with many brewer's and distiller's looking to gain value from their co-products. The production of ethanol from SG is an avenue that could potentially be exploited.

Additionally, the project has shown that the use of ultrasound in the conversions of lignocellulose has the potential to improve the process, both in terms of increasing sugar yields during enzymolysis, as well as being a promising technology for low energy pre-treatment. Reductions in enzyme dosing and energy inputs during the production of bioethaol from lignocellulose are opportunities which could be exploited by the emerging cellulosic bioethanol industry and this has particular relevance to the American bioethanol industry which utilises maize based feedstocks and generates co-products similar to the maize based SG used in this study. Ultrasound has already been shown to be scalable to industrial levels, with recent implementation within the anaerobic digestion and waste-water treatment industries (Webber Ultrasonics GmbH, 2013).

8.6 Suggestions for future work

Until now, little has been known about the effects of ultrasound upon ozonolysis of lignocellulose. This work has shown that ultrasound and O_3 and ultrasound, O_3 and H_2O_2, are effective in the pre-treatment of lignocellulose. However, it is not as effective as established conventional

methods such as pre-treatment with acid. Whilst the research presented here has gone some was to giving further insight into the use of ultrasound in the pre-treatment of lignocelluloses, it did little to evaluate the possible link between the efficiency of ultrasonic pre-treatment and the levels of hydroxyl radical production within the reactor. In addition to this the work presented here did little to assess possible reductions in fermentation inhibitor production during ultrasonic pre-treatment. The lower temperatures utilised during ultrasonic pre-treatment might be expected to reduce inhibitor production compared to high temperature acid treatment.

Therefore it is proposed that further work should seek to further optimise the use of ultrasound in the pre-treatment of lignocellulose and investigate the potential link between hydroxyl radical production and the effectiveness of pre-treatment. In addition to this optimised systems for ultrasonic pre-treatment should be assessed in terms of their potential for reduced fermentation inhibitor production. This could be approached in a number of ways, including: 1) Repeating ultrasound and hydrogen peroxide pre-treatment studies with varying concentrations of hydrogen peroxide, quantifying hydroxyl radical production and evaluating its effect upon pre-treatment, 2) Repeating ultrasound, ozone and hydrogen peroxide pre-treatment studies with varying concentrations of ozone and hydrogen peroxide, quantifying hydroxyl radical production and evaluating its effect upon pre-treatment, and 3) HPLC characterisation of fermentation inhibitors produced by ultrasonic pre-treatment, alongside quantification of lignin breakdown products to give a greater understanding of lignin degradation during ultrasonic pre-treatment.

Whilst research documented in this thesis has expanded knowledge in terms of the effects of ultrasound upon hydrolytic enzyme function, there are still a large number of areas in this research field which are poorly understood. As such, it is proposed that further work should seek to investigate: 1) The mechanism by which ultrasound increases enzyme function, 2) The reasons pertaining to why optimal frequencies for accelerating enzyme function differ dependent upon substrate characteristics.

The effects of ultrasound upon biological processes are a research area which is in its infancy. To date much of the work in this area (including that detailed in this thesis), has concentrated on assessing the effects of ultrasound upon enzyme function. However, the effects of ultrasound upon biological entities at the level of the cell, are an area that is poorly understood. As such, a further area which warrants investigation is the effects of ultrasound upon fermenting yeasts.

Chapter 9

Concluding discussion

9. References

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